STUDIES ON THE SURFACE ANTIGENS AND SURFACE PROPERTIES OF ANIMAL CELL HETEROKARYONS

A thesis presented for the

Degree of

Doctor of Philosophy

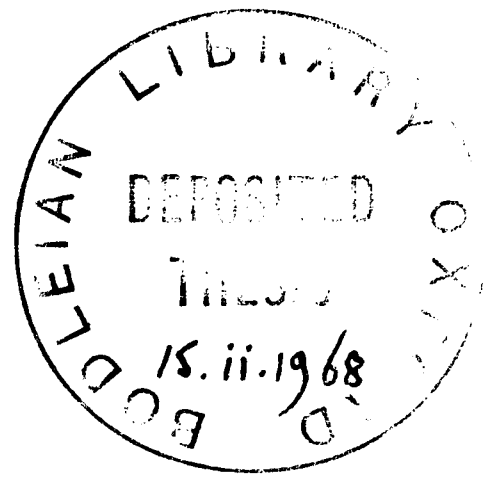

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TABLE OF CONTENTS

(Introduction - continued) Page

(Materials and Methods - continued) Page

(Results - continued) Page

(Discussion - continued) Page

ABBREVIATIONS

AES - Anti-Ehrlich serum

AHS - Anti-HeLa serum

ALS - Anti-L serum

HAU - Haemagglutinating units

PBS - Phosphate buffered saline

INDEX OF FIGURES

INDEX OF TABLES

PHOTOGRAPH NEGATIVE NUMBERS

Graphs and diagrams - Z56 to 59

Figure	Negative Number
3	10/32
4	8/33
5	14/5
6	19/22
9	6/3
10	6/4
12	2/2
13	1/27
14	6/8
17	23/26
18	23/28
19	L2938
20	22/6
22	JFW
23	CP64/5
24	21/14
25	JFW
26	2/40
27	21/15
28	7/39
29	13/42
30	13/3
31	L2952
32	CP66/2
33	L2953
34	11/2
35	5/28
36	4/20
37	2/1
42	L2978
43	L2977
44	L2976
45	L2979
46	12/10
48	18/6
49	24/20
50	CP66/4
51	20/32
52	16/37
53	CP64/6
55	5/33
56	20/31
57	16/34
58	16/35
59	H11/25
62	CP62/1
63	1/26
66	CP62/0
67	CP65/7
71	63/10
73	9/40
74	9/1
75	9/41
76	14/7
77	3/17
78	19/21
79	4/21
80	1/24
81	1/23
82	15/42
83	7/38

INTRODUCTION

1. Heterokaryons produced with UV-inactivated Sendai virus

Heterokaryons produced by viral fusion of cells of different species were first described by Harris and Watkins (1965). HeLa cells of human origin and Ehrlich ascites tumour cells from the mouse were fused with Sendai virus inactivated by ultraviolet light. Each type of nucleus was capable of DNA and RNA synthesis, and the heterokaryons synthesized protein. Fusion of different nuclei to give heterosynkaryons could occur, since some metaphase figures contained chromosomes of each species. Confirmation of hybrid mitosis was provided by Harris, Watkins, Campbell, Evans and Ford (1965). They suggested that nuclear fusion was due to synchronous mitosis of the component nuclei. Most heterokaryons did not give rise to viable progeny, and this was attributed to the inability of single Ehrlich ascites cells to grow in vitro. Binucleate heterokaryons (dikaryons) appeared to be the only hybrid cells capable of continued multiplication, since the few hybrid mitoses seen some days after cell fusion contained two chromosomal sets. Further study was prevented by culture overgrowth with single HeLa cells.

Harris (1965) fused HeLa cells with rat thoracic duct lymphocytes, rabbit macrophages and hen erythrocytes which are nucleated. In heterokaryons the nuclei of these differentiated cells enlarged and synthesized

DNA and RNA. It was noted that the immunologically competent lymphocytes did not seem to affect the viability of heterokaryons over a period of days. This work was summarized by Harris, Watkins, Ford and Schoefl (1966). When a cell which synthesizes DNA or RNA is fused with one which does not, the active partner always induces nucleic acid synthesis in the inactive partner. Further work on HeLa-hen erythrocyte heterokaryons by Harris (1967) suggested that the synthesis of RNA by the hen nuclei was secondary to nuclear enlargement. The pattern of disappearance of nuclear bodies suggested that nuclear enlargement might control not only the amount of RNA synthesized but also the areas of chromatin on which it was synthesized. Signals for nuclear enlargement and RNA synthesis seemed to have a low order of specificity.

2. Reasons for studying the surface antigens of heterokaryons

These very interesting results all concerned intracellular events, but the nature of changes at the surface of heterokaryons remained a mystery. It had been noted by Harris and Watkins (1965) that HeLa-Ehrlich heterokaryons had the HeLa surface property of sticking to glass. It was not known if Ehrlich antigens were still present and still produced in these heterokaryons. A study of the surface antigens of these cells would be of assistance in understanding the general properties of cell surfaces and cell membranes. In addition the presence or absence of

antigens might provide information on protein synthesis by each component of a heterokaryon. This is of particular interest and importance in heterokaryons formed by fusing HeLa cells with hen erythrocytes which in their normal state do not synthesize DNA, RNA or protein. Would synthesis of RNA by the hen nucleus in a heterokaryon be enough to initiate the synthesis of hen antigens, and would such antigens be detectable on the surface of heterokaryons?

Methods of identifying surface antigens could also have other applications. After a few days in culture it may be very difficult to recognize heterokaryons because of nuclear enlargement or nuclear fusion. The relative increase of single cells, for example the overgrowth of cultures containing HeLa-Ehrlich heterokaryons by single HeLa cells, as described by Harris and Watkins (1965), also makes it difficult to follow heterokaryons for any length of time. Antigenic marking could therefore be of assistance in metabolic studies and might also help in identifying and isolating a hybrid cell line if hybrid growth were to occur. For these reasons a study was carried out on the properties of heterokaryon surfaces, with particular reference to the behaviour of surface antigens.

3. The cell membrane

Before considering the methods and problems of antigen labelling in

tissue culture, the general properties of the cell membrane and cell surface will be reviewed. Processes in which there is cell surface contact and recognition will also be discussed. Some of the work may not seem of direct relevance to a study on the surface antigens of heterokaryons, but must in fact be considered as one attempts to understand the interaction of two cell membranes with differing antigens and surface properties. It may also be of value to have a review of this subject at a time when the mammalian cell surface is attracting an increasing amount of attention.

(i) History

The origin of the plasma membrane concept has been attributed by Smith (1962) to the German worker Carl Nägeli. Nägeli (1855) noted the impermeability of the plant cell surface and concluded that it was responsible for the osmotic properties of cells. Pfeffer (1877) showed the similarity in behaviour of plant cells and artificial osmometers and postulated the presence of an invisible plasma membrane. The importance of lipids in the cell membrane was demonstrated by Overton (1899) using cell osmotic volume changes as a measure of the rate of entrance of various solutes into living cells. The work of Höber (1910, 1926) supported the cell membrane concept. He found that intact erythrocytes had a high electrical resistance while the interior of the cell and the medium both conducted electricity well.

The name of Chambers is also important in early studies on the cell membrane. He found (1922-23) that the membrane of invertebrate eggs is not penetrated by intracellular dye, and his work on the penetration of oil drops into invertebrate eggs (Chambers, 1935; Chambers and Chambers, 1961) contributed to the understanding of membrane permeability. Mudd and Mudd (1926, 1931) investigated the ease with which cells were wetted by and coalesced with a film of oil. From the fact that erythrocytes were readily wetted while leucocytes had a hydrophilic surface, they deduced that the leucocyte is coated with protein while the erythrocyte surface is composed of lipid and protein.

The first suggestion that the cell membrane was bimolecular came from the classic experiments of Gorter and Grendel (1925). They spread lipid extracted from erythrocytes as a unimolecular film in a trough designed by Langmuir (1917). The area of the film measured about two times that of the total surface area of the red cells. The low surface tension at the surface of cells led Danielli and Harvey (1935) to suggest that lipid polar surfaces must be covered by at least one layer of protein. Danielli and Davson (1935) proposed that the cell membrane was a bimolecular phospholipid leaflet between two layers of protein, and this has been the basic membrane model for

many years. The protein on the surface of the cell is thought to exist in two forms: globular protein which can be removed from the membrane simply by washing, and native protein which can be removed only by denaturation of the phospholipid membrane (Brown and Danielli, 1964).

(ii) Myelin and the unit membrane

Current concepts of the cell membrane have been partially based on the study of myelin. The general pattern of concentric lipid-protein layers was first described by Schmitt (1950) basing his ideas on the X-ray diffraction studies of Schmitt, Bear and Clark (1935) and the polarized light studies of Schmidt (1936, 1937). This was confirmed in the electron microscope observations of Fernández-Morán (1950, 1952). Geren (1954) showed that myelin develops directly from the surface of the Schwann cell. Sjöstrand and Rhodin (1953) described the double layer appearance of cell membranes under the electron microscope, but it was the work of Robertson (1957, 1959) which stressed the similarity in structure of various cell membranes. Electron micrographs of myelin and the surfaces of a wide variety of cells showed a membrane unit about 75 Å wide in which two parallel dense bands 25 Å thick were separated by a light zone 25 Å wide. Such a unit membrane was seen in electron micrographs of mitochondria,

endoplasmic reticulum and nuclear membranes.

Many writers in recent years (Fawcett, 1962; Korn, 1966) have criticized electron microscopic studies and the unit membrane concept because they tend to give the idea that all cell membranes are similar. These ultrastructural studies artifically emphasize the lipoproteins of membranes without taking into consideration the protein enzymes and external coatings which are so important in membrane function. The fact that proteins play an important but poorly understood role in membrane structure has been stressed by Hechter (1965), Wallach and Zahler (1966) and Weiss (1963). It is felt by Korn (1966), Green and Perdue (1966) and others that myelin is not at all representative of cell membranes in general. Myelin is metabolically inert (Davison and Dobbing, 1960) and has a very low ratio of protein to phospholipid in comparison with other cell membranes (Ashworth and Green, 1966).

(iii) Structural variations

The basic phospholipid bimolecular layer may not be a valid generalization, since membranes could be composed of lipid micelles under some conditions (Finean, 1966; Korn, 1966; Lucy, 1964; Lucy and Glauert, 1964) or of repeating units (Blasie, Dewey, Blaurock and Worthington, 1965; Green and Perdue, 1966) and could alternate between a bimolecular lipid leaflet and a micellar form (Kavanau, 1963).

(iv) Biochemistry

Many ideas on the composition of the cell membrane have come from studies on the erythrocyte ghost. Parpart and Dziemian (1940) showed that phospholipids and cholesterol constitute about 90% of the red cell lipids and the ratio of lipid to protein was 1 to 1.7. However, Ponder (1961) is doubtful that the erythrocyte ghost really represents the red cell membrane, and the data would be even less applicable to other cells. The membranes of nucleated cells can be isolated only by cell homogenization or other traumatic procedures, and it is extremely difficult to obtain pure preparations (reviewed by Maddy, 1966).

(v) Summary

The structure of the cell membrane is not well understood. The generally accepted view is that it is a bimolecular lipid leaflet coated on each surface with protein, but this concept has been subject to recent modification. Under the electron microscope all cell membranes have a similar appearance, but chemically and functionally they show considerable diversity.

4. The cell surface

(i) Evidence for a surface layer

Evidence for the presence and role of a cell surface layer is

no less confusing a subject than the structure and chemistry of the actual cell membrane. Weiss (1963) points out the difficulties involved in studying the cell surface and suggests that there are extra-membranous materials, such as hydrated carbohydrate-containing mucoids, which must be considered functionally as the cell surface without necessarily being regarded as membrane components. In the Davson-Danielli model the cell membrane is coated with protein, and Robertson (1964) suggested that the surface coat was mucoprotein or mucopolysaccharide. In earlier work (Robertson, 1958) he described a 100 to 150 Å gap between adjacent cell membranes in myelin. The thickness of this gap could be altered by solutions of different tonicities. This was felt to support the concept of a surface layer. Such a gap has been demonstrated in vivo between mouse kidney cells (Miller, 1960) but is of low viscosity, since haemoglobin molecules will diffuse between cells. Ambrose and Jones (1961) showed with the surface contact microscope that tissue culture cells appear to be separated from glass surfaces. This space may represent a surface layer.

(ii) Non-vertebrate cells

As pointed out by Davson (1962) surface coats do surround many cells. Polysaccharides form the cellulose walls of plants and the capsules and slime coats of bacteria. There are external coats about

many invertebrate eggs (Chambers and Kopac, 1937; Chambers, 1940) and about the cells of amphibian embryos (Holtfreter, 1943). A surface mucopolysaccharide layer is found about amoebae (Bairati and Lehman, 1953; Pappas, 1954) and filamentous extensions of the amoeba cell surface can be seen under the electron microscope (Pappas, 1959). Such a surface layer is important in binding protein from the medium to the cell surface, and this has been demonstrated by a variety of methods (Schumaker, 1958; Brandt, 1958; Brandt and Pappas, 1960; Brandt, 1962).

(iii) Vertebrate cells

It may not be wise to apply these results to mammalian cells because of great differences in cell structure and function. There is however mounting evidence for a surface layer, which seems to be mucopolysaccharide in nature, about many mammalian cells, and this could be of importance in membrane function (Bennett, 1963; Fawcett, 1965). Such a layer has been shown at the surface of gall bladder epithelium (Yamada, 1955), intestinal epithelium (Palay and Karlin, 1959), urinary bladder (Peachey and Rasmussen, 1961; Choi, 1963), thyroid cells (Seljelid, 1967), kidney tubule cells (Seljelid and Ericsson, 1965), and recently at the surface of a great variety of mammalian cells (Rambourg et al., 1966; Rambourg and Leblond, 1967). The blood

group antigens themselves are examples of surface mucopolysaccharides (Morgan and Watkins, 1959). Finean (1966) feels, however, that there is no direct evidence that mucopolysaccharide is an integral part of the cell surface or is responsible for holding apart the dense components of opposed cell membranes.

(iv) In vitro studies

In vitro studies would suggest that there is a surface layer about some cells. Rosenberg (1960) detected a 'microexudate' about tissue culture cells allowed to settle on clean glass, and he ascribed a variety of roles in cell locomotion, adhesion, and interaction to this layer. Weiss (1958) showed that mouse ascites cells lost 20% of their dry weight after trypsin treatment without any decrease in viability or change in diameter. This suggested the presence of extracellular substances, though a mucopolysaccharide surface layer could not be demonstrated by staining. Daniel, Dingle, and Lucy (1961) showed that repeated washings of a monolayer of rat fibroblasts contained no hyaluoronic acid but that trypsinization of the cells released considerable amounts of mucopolysaccharide. This may indicate material ordinarily associated with the cell surface. Surface mucopolysaccharides have been demonstrated histochemically on mouse ascitic mammary carcinoma cells (Gasic and Gasic, 1962) and polyoma transformed

hamster cells (Defendi and Gasic, 1963). Cook, Heard, and Seaman (1960) released a sialomucoprotein from the surface of erythrocytes with trypsin and an extracellular layer has been found about chick embryo cells (Lesseps, 1967). It has been suggested that ribonucleic acid may be present in or near the cell surface (Weiss and Mayhew, 1966).

(v) Summary

The cell membrane is coated with an outer layer of protein. A surface mucopolysaccharide layer has been demonstrated about many invertebrate and some mammalian cells, but the general presence and role of such a layer is still controversial.

5. Cell interaction in vitro

(i) Reasons for consideration

The cell surface is of course of great importance in the interaction of cells in vivo. An example is the study of Gesner and Ginsberg (1964) in which glycosidase treatment of lymphocytes in vitro resulted in failure of living transfused cells to reach the spleen. This was felt to be due to an alteration in cell surface sugars. Certain in vitro processes involving cell surface contact and recognition have received particular attention in recent years, and these are the aggregation of embryonic cells, contact inhibition of cells in tissue culture,

syngeneic preference, and destruction of tissue culture cells by non-immune lymphoid cells. This behaviour, resulting from cell surface contact, may be compared with the behaviour of heterokaryons in which there is cell surface mixing.

(ii) Aggregation of embryonic cells

The reaggregation of dissociated amphibian embryos was noted by Roux (1894). Wilson (1907) showed that sponges squeezed through fine cloth could reaggregate to give functional sponges. Moscona (1956) demonstrated that chick embryo cells would reform tissue-like groupings which would develop in accordance with the tissue of origin. The fact that reaggregation was decreased at low temperatures (Moscona, 1961a and 1961b) and by puromycin (Moscona and Moscona, 1963, 1966) was felt to indicate that the synthesis of cell surface products was required for aggregation. A glycoprotein capable of giving selective reaggregation of dissociated sponge cells has been isolated (Margoliash, Schenck, Hargie, Burokas, Richter, Barlow and Moscona, 1965). Steinberg (1963) criticized Moscona's concept of an extracellular material (ECM) necessary for cell aggregation and showed that the viscous material referred to as ECM was really a highly hydrated deoxyribonucleoprotein gel formed from the chromosomes of ruptured cells due to the action of trypsin. Weiss

and Kapes (1966) feel that Steinberg's work is correct but that the presence of gaps of low electron density between adjacent cells and the change in cell charge after trypsin treatment support Moscona's basic concept of an ECM. Such a material could affect the fusion of embryonic cells and the behaviour of the heterokaryon surface after fusion.

(iii) Contact inhibition

There is inhibition of movement and growth of normal cells in confluent cultures. Abercrombie and Heaysman (1953, 1954) described inhibition of movement caused by contact between normal fibroblasts. Normal cells did not decrease the movement of sarcoma cells (Abercrombie and Heaysman, 1954b; Abercrombie, Heaysman and Karthauser, 1957). Malignant cells appeared to have escaped from 'contact inhibition'. Loss of contact inhibition of movement is particularly characteristic of cells transformed by virus or other agents (Stoker, 1964a; Stoker, Shearer and O'Neill, 1966). Inhibition of growth in confluent cultures is not well understood. There is a marked decrease in DNA, RNA and protein synthesis (Levine, Becker, Boone and Eagle, 1965), but the phenomenon is influenced by changes of medium (Todaro, Lazar and Green, 1965). Escape from contact inhibition of growth is a characteristic feature distinguishing heteroploid

from diploid cells (Eagle and Levine, 1967). It is difficult to predict what influence contact inhibition of movement and growth could have on the morphology and viability of heterokaryons formed by fusing cells with different surfaces.

(iv) Syngeneic preference (allogeneic inhibition)

Another interesting phenomenon which apparently results from cell surface contact is syngeneic preference. The fact that mouse parental tumour grafts grow poorly in an F_1 hybrid in spite of the absence of an immunological reaction was noted by Snell (1958)and further investigated by Hellström (1963). Syngeneic preference has been postulated as an in vivo control mechanism by which cells with an altered surface structure can be eliminated without the need for antigen-induced stimulation (Hellström and Hellström, 1966; Klein, 1966). Mouse tumour cells in vitro grow more poorly after exposure to extracts of allogeneic cells than after exposure to extracts of syngeneic cells (Hellström, Hellström and Haughton, 1964). Tumour growth in syngeneic hosts is decreased by exposure of tumour cells in vitro to cells or cell extracts bearing foreign H-2 antigens (Hellström, Hellström and Bergheden, 1965; Möller and Möller, 1967). However, this phenomenon occurs only with small numbers of tumour cells and with some tumours may not occur at all (Wallace, 1965). In some cases it may also be abolished by radiation which may indicate an

immunological basis (Oth and Burg, 1965; Sanford, 1967).

(v) Cell destruction by non-immune lymphocytes

A similar phenomenon has been described by Möller and Möller (1965a) in which normal non-immune mouse lymphoid cells are capable of destroying allogeneic tissue culture cells if the lymphocytes are aggregated to the tissue culture cells by substances such as phytohaemagglutinin or inactivated antiserum. The fact that this effect occurred with irradiated lymphocytes and that F_1 lymph node cells could kill parent tumour cells suggested that the action was not immunological (Möller and Möller, 1965b) and might also be an in vivo control mechanism for eliminating cells with altered surface structure. It is interesting to speculate whether these phenomena bear any relationship to the viability and behaviour of heterokaryons formed with antigenically different cells and with lymphocytes.

6. Other cell properties dependent on the cell surface

(i) Adhesion

The adhesion of cells to each other in vivo and to glass surfaces in vitro is another field in which the role of the cell surface is poorly understood. Curtis (1962) discusses some factors which could be important in cell adhesion: ionic bonds in which calcium may be important, covalent bonds such as disulphide bridges, hydrogen bonds,

and long range van der Waal's forces. Coman (1944) showed that there was a decrease in the mutual adhesiveness of malignant cells and suggested that this might be due to a decrease in surface calcium. The use of the chelating agent EDTA to disperse some types of cells (Rinaldini, 1959) may indicate the importance of calcium in adhesion. The fact that trypsin can be used to disperse cells has been known for many years (Rous and Jones, 1916). The action of trypsin on the peptide linkages adjacent to arginine and lysine would appear to implicate cell surface protein in cell adhesion in vitro (Moscona, Trowell, and Willmer, 1965).

It is known that serum proteins may assist cell adhesion in culture (Lieberman and Ove, 1958) and fetuin of foetal calf serum has been studied in this regard (Fisher, Puck, and Sato, 1958). Although some cells can be adapted to growth in synthetic medium without added protein (Rappaport, Poole, and Rappaport, 1960), serum seems necessary for the prolonged growth of most cells in vitro. However, cells washed in protein-free medium and allowed to settle from such a medium will adhere to glass much more rapidly than cells in protein-containing medium (Easty, Easty, and Ambrose, 1960; Taylor, 1961). This effect does not require cell activity and is observed with formalin-treated cells. It does not seem to be clear why some types of cells,

such as HeLa cells, adhere to glass, spread out, and grow well in vitro while others, such as many lines of Ehrlich ascites cells, adhere to glass poorly and do not grow in vitro, although cells which show a high net negative charge also adhere poorly to glass (Ambrose, 1966).

(ii) Movement and morphology

Cell morphology in vitro may depend on cell surface properties (Willmer, 1965) but neither morphology nor the related subject of cell movement are well understood. Simple sugars in the culture medium may influence the morphology of tissue culture cells, perhaps due to an effect at the cell surface (Cox and Gesner, 1965). Leucocytes are attracted by chemotaxis (Harris, 1954), but this does not seem to be a factor in the movement of most cells in tissue culture. In vitro studies with the cell contact microscope and with interference microscopy (Ambrose, 1956; Abercrombie and Ambrose, 1958; Abercrombie, 1961) have shown that fibroblasts are preceded by a ruffled, undulating membrane, and that there are ruffles on both the top and bottom surfaces of the cell which pass backwards as transverse waves.

Ambrose (1961) feels that the main locomotory mechanism of fibroblasts on a solid substrate is undulation of the cell membrane in the region adjacent to the substrate. Carter (1965) feels that cells tend to move in the direction of increasing adhesion to the substrate. These

studies also emphasize the constant motion of the surface of cells in tissue culture, a fact which was recognized many years ago (Lewis and Lewis, 1924). Cell movement is not immediately dependent on nuclear direction, since movement may be shown by enucleate fragments of human cells (Goldstein, Cailleau, and Crocker, 1960) and enucleate amoebae (Comandon and de Fonbrune, 1939). From these results it would be difficult to predict what morphology and movement might be shown by heterokaryons after the fusion of different types of cells.

(iii) Surface charge

The nature of various ionic groups at the cell surface is not well known, although the overall charge can be studied by cell electrophoresis. Cells bear a net negative charge (Furchgott and Ponder, 1941), and the carboxyl groups of sialic acid make a major contribution to this charge. This has been demonstrated on erythrocytes (Cook, Heard, and Seaman, 1961; Eylar, Madoff, Brody, and Oncley, 1962), ascites tumour cells (Cook, Heard, and Seaman, 1962; Wallach and Eylar, 1961) and on both by Ruhenstroth-Bauer, Kübler, Fuhrmann, and Rueff (1961). Tumour cells bear a higher negative charge than normal cells (Ambrose, James, and Lowick, 1956; Lowick, Purdom, James, and Ambrose, 1961). This is true for virally transformed

cells (Forrester, Ambrose, and Macpherson, 1962), and the amount of charge is related to the degree of malignancy (Purdom, Ambrose, and Klein, 1958). However, increased surface charge may be a general phenomenon associated with a rapid rate of cell division (Eisenberg, Ben-Or, and Doljanski, 1962; Heard, Seaman, and Simon-Reuss, 1961). Cell electrophoresis would be an interesting technique to apply to hybrid cells if a viable line could be obtained from cells differing in electrophoretic mobility.

7. Identification of surface antigens in vitro.

(i) Fluorescent and ferritin-labelled antibody

Many methods are available for identifying the antigens of cells. In recent years the fluorescent antibody technique (Coons, Creech and Jones, 1941) and the ferritin-labelled antibody method (Singer, 1959) have been used extensively. However, these methods have certain disadvantages. Conjugation of the fluorescein or ferritin label with antiserum is time-consuming, large quantities of antiserum are required, specificity is difficult to obtain, and the methods are not particularly sensitive. Moreover, these methods are not suited to the study of living cells on glass coverslips, since the cells must be killed for examination. Ferritin-labelled antibody can only be identified with the electron microscope. Cellular stains and fluorescent antibody

cannot be used simultaneously, and therefore accurate identification of the component nuclei of heterokaryons would be difficult.

(ii) Mixed agglutination

For the above reasons it was felt that methods using marker particles, such as erythrocytes, would be more suitable for antigen identification. Three such techniques were considered: mixed agglutination, immune adherence and mixed haemadsorption.

Mixed agglutination occurs when different particles such as erythrocytes and tissue cells are linked together by antibody to a common antigen. Wiener and Herman (1939) observed this type of reaction when pneumococci and human erythrocytes formed mixed aggregates with horse antiserum to pneumococci. The technique has been used frequently in recent years to identify the surface antigens of cells (Coombs, Bedford, and Rouillard, 1956; Coombs, Daniel, Gurner, and Kelus, 1961 a, 1961 b; Franks, Coombs, Beswick, and Winter, 1963). Mixed agglutination is not very sensitive but does have the great advantage that specific antigens, for example blood group antigens, can be identified on tissue cells.

(iii) Immune adherence

Immune adherence was apparently first observed by Duke and Wallace (1930), although a similar phenomenon was described much

earlier (Levaditi, 1901). The technique has been investigated in recent years by Nelson (1953, 1956) and reviewed by Turk (1964). Human and primate red cells and some types of platelets will adhere to the site of a complement-fixing antigen-antibody reaction in the presence of calcium and magnesium ions and at a temperature of 37°C. This type of method has been used under the name of serological adhesion by Kano and Milgrom (1965) for studying the surface antigens of cells in tissue culture. It is quite sensitive.

(iv) Mixed haemadsorption

This method was first used by Fagraeus and Espmark (1961) for identifying virus antigens on infected tissue culture cells. The principles are based on the mixed antiglobulin method of Coombs, Marks, and Bedford (1956) and Chalmers, Coombs, Gurner, and Dausset (1959) in which different types of cells sensitized with antibody from one species are linked by an antiglobulin antibody. The technique, given various names, has been used to detect species antigens (Milgrom, Kano, Barron, and Witebsky, 1964), blood group antigens (Edwards, Ferguson, and Coombs, 1964; Kano, 1966), antibodies present after viral infection (Kano, Milgrom, Barron, and Witebsky, 1965), iso-antigens (Metzgar, Flanagan, and Zmijewski, 1965), antibodies present after tissue transplantation in mice (Abeyounis, Milgrom, and Witebsky,

1964), rabbits (McDonald, Milgrom, Abeyounis, and Witebsky, 1965) and humans (Milgrom, Kano, and Witebsky, 1965), IgM antibodies (Jonsson, Espmark, and Fagraeus, 1966), isoantibodies following blood transfusion (Kano and Milgrom, 1967) and histocompatibility and tumour virus antigens (Barth, Espmark, and Fagraeus, 1967). The technique is very sensitive, giving positive reactions with antiserum diluted to 1 in 1,000,000 (Barth et al., 1967). The antiglobulin type of reaction is one of the most sensitive immunological methods in use and may detect as little as 0.0045 μg of antibody N/ml (Marrack, 1963). There are several disadvantages, however. The doubly sensitized erythrocytes used as indicator particles will detect any antibody on the cell surface, and it is difficult to obtain sufficiently pure antisera for the detection of specific antigens. Also, heterologous antiserum to one cell type may cross react extensively with other cells (Espmark and Fagraeus, 1965).

(v) Viral haemadsorption

Methods employing red cells are suitable for identifying antigens, but studies on heterokaryons could be complicated by viral haemadsorption. It has been known for some time that myxoviruses will agglutinate erythrocytes (Hirst, 1941) and that virus-infected cells may be identified by haemadsorption (Vogel and Shelokov, 1957). Moreover, viral haem-

agglutinin will persist for a few days on the surface of heterokaryons (Harris and Watkins, 1965), the degree of haemadsorption depending on the amount of virus used for cell fusion. A possible solution to this problem was suggested by the work of Hirst (1941, 1942) in which it was shown that agglutinated erythrocytes would elute virus particles after a period of time at 37^{o}C.

(vi) Double labelling

Antigen labelling on heterokaryons by a single haemadsorption technique would be adequate for many experiments. For example, single Ehrlich cells, hen erythrocytes, and rat lymphocytes do not stick to or grow on glass. If these cells were fused with HeLa cells, any mononucleate or multinucleate cell bearing Ehrlich, hen or rat antigen would be a hybrid cell. Complications arise when two cells, each of which stick to glass, are fused with each other. An example is HeLa cells and mouse L strain fibroblasts. A mononuclear cell labelled for L antigen could be a single L cell, a homosynkaryon (fused L cell nuclei) or a heterosynkaryon (fused HeLa and L nuclei). Some sort of double labelling technique was therefore necessary.

Most of the double labelling experiments reported to date have employed fluorescent antibodies and have not been particularly successful. White (1958) used antibody labelled with fluorescein

(yellow) and rhodamine (red) in an attempt to demonstrate two types of antibody in the same plasma cell. The same type of technique has been applied to single antibody-forming cells by Hiramoto and Hamlin (1965) and Green, Vassalli, Nussenweig, and Benacerraf (1967) and to the surface antigens of paramecium by Beale and Mott (1962). A different type of double labelling has been carried out using globulin labelled with fluorescein and ferritin (Levinthal, Wicker, and Cerottini, 1967). These studies have the disadvantages of the fluorescent antibody and ferritin techniques that were mentioned earlier and cannot be applied to living cells on a glass surface.

A more profitable approach seemed to be the use of a particle visually distinct from erythrocytes. Bacterial adherence has been used to detect antibody forming cells (Makela and Nossal, 1961). Latex particles have been used to identify blood group antigens on cells in tissue culture (Hagiwara, 1962) and to detect antibody forming cells (Hagiwara and Muramatsu, 1963). Tissue culture cells have been marked by India ink ingestion (Stoker, 1964 b) and antibody forming cells detected with antigen-coated bentonite particles (Baker, Bernstein, Pasanen, and Landy, 1966). Fagraeus, Espmark, and Jonsson (1965) suggested that bacteria or fungi might be doubly sensitized and used in place of doubly sensitized erythrocytes in the mixed haemadsorption

reaction. Of the particles mentioned above, bacteria seemed to be the most convenient in terms of size and availability. Moreover, anti-bacteria antisera are easily obtained and therefore bacteria could be used in an antiglobulin type of method, although this had apparently never been done.

(vii) Cytolysis

Another means of identifying surface antigens on cells in tissue culture is with cytolytic antibody and complement. The cytolytic effect of antiserum has been known for many years. Lambert and Hanes (1911) demonstrated that plasma from immunized animals could prevent tumour growth in vitro and Niven (1929) clearly described the cellular effects of cytotoxic antibody in vitro. Since that time there have been hundreds of studies on the morphological and biochemical changes produced in mammalian cells with isologous and heterologous antiserum and complement. The production of functional holes in the cell membrane by antibody and complement was suggested by Green, Barrow, and Goldberg (1959), and such lesions were demonstrated in the erythrocyte and ascites cell membranes in an electron microscope study by Humphrey and Dourmashkin (1965). As discussed below, cytolysis of cells with specific antibody and complement has been used to demonstrate species antigens on the surface of cells in tissue culture.

8. Behaviour of surface antigens in vitro

(i) Species antigens

The retention of species antigens is a characteristic feature of cells in tissue culture. This has been shown with cytolytic antiserum (Goldstein, 1957; Defendi, Billingham, Silvers, and Moorhead, 1960; Goldstein and Myrvik, 1960), by mixed agglutination (Brand and Syverton, 1960; Coombs et al., 1961 a, 1961 b; Franks et al., 1963), with fluorescent antibody (Stulberg, Simpson, and Berman, 1961) and by agar diffusion (Brand and Chiu, 1966 a, 1966 b). This is to be contrasted with the nutritional and metabolic similarity of many cells in tissue culture (Levintow and Eagle, 1961) and the alterations which take place in chromosomes after prolonged culture (Hsu, Billen, and Levan, 1961).

(ii) Tissue-specific, blood group, and Forssman antigens

The retention of species antigens must also be contrasted with the loss of some organ-specific antigens (Weiler, 1959; Forbes, Roitt, Doniach, and Solomon, 1962; Fogel, 1963; reviewed by Dumonde, 1966). This loss may be accompanied by an increase in Forssman antigen (Fogel and Sachs, 1962, 1964 a, 1964 b), and in some cases tissue-specific antigens may be retained in tissue culture (Rosenau and Moon, 1961 a). Blood group antigens may be lost from cells in culture

(Högman, 1960), although H antigen has been shown on HeLa cells (Kelus, Gurner, and Coombs, 1959) and blood group antigens on monkey kidney cells (Franks et al., 1963; Kano, 1966). To make matters more complicated, one cell can apparently give rise to a mixed population in which some cells carry blood group A antigen and some do not (Franks and Dawson, 1966). Forssman antigen can also cause complications in antigen studies in tissue culture (Franks, Daniel, Gurner, and Coombs, 1964). Established cell lines from the Forssman-positive mouse may be negative for Forssman antigen, while cell lines from the Forssman-positive dog and Chinese hamster continue to carry Forssman antigen. Moreover, negative cells may become positive in serum containing Forssman antigen. Other surface alterations during primary culture of cells include changes in virus susceptibility (Holland, 1961; Pulvertaft, Davies, Weiss, and Wilkinson, 1959), appearance of new or previously undetected antigens (Sanford, Merwin, Hobbs, Fioramonti, and Earle, 1958; Fogel, 1965; Holmgren and Payne, 1966; Timbury, 1963) and changes in the concentration of existing antigens during long-term culture (Coriell, Fall, and Gaskill, 1958). Embryonic cells show a decreased capacity to reaggregate during the first few days in culture (Moscona, 1960).

(iii) Genetic control of surface antigens

Very little is known about the genetic control and chemistry of cell surface antigens. Much of the genetic knowledge comes from studies of the histocompatibility antigens of highly inbred strains of mice (Snell, 1948). There are at least 15 histocompatibility loci in mice (Billingham, Hodge, and Silvers, 1962; Barnes and Krohn, 1957) and at least 20 alleles involved in the complex H-2 system (Amos, Gorer, and Mikulska, 1955; Gorer and Mikulska, 1959). The antigens are associated with the membrane systems of the cell, particularly the cell surface (Herzenberg and Herzenberg, 1961; Herbermann and Stetson, 1965; Haughton, 1966). The genetic control and distribution of species antigens in the cell is not well understood although, as previously mentioned, cell surface species antigens are retained in tissue culture in spite of chromosomal, enzymatic and nutritional changes in the cells. A considerable amount of work has been done on the surface antigens of paramecium stemming from the studies of Sonneborn (1937) and it has been known for some time that alterations in surface antigens depend on cytoplasmic changes (Sonneborn and LeSuer, 1948). The pattern of possible antigens in paramecium is controlled by the nucleus, but the particular antigen expressed depends on cytoplasmic factors which are influenced by the environment (Beale, 1958). The situation is

different in amoeba where nuclear transplantation studies have shown that the nucleus controls surface antigens but the cytoplasm controls the morphology of the cell. (Danielli, Lorch, Ord, and Wilson, 1955).

(iv) Chemistry of surface antigens

Until recent years most chemical studies on surface antigens have been confined to bacteria (reviewed by Salton, 1964), paramecium (Preer, 1959 a and 1959 b; Beale and Jones, 1963; Steers, 1965), and to the human blood-group antigens (Kabat, 1956; Morgan and Watkins, 1959). The greatest problem in studying mammalian surface antigens has been in obtaining a suitable soluble preparation. Soluble H-2 antigens have been obtained from the ascitic fluid associated with mouse tumours and found to have a lipoprotein structure (Davies, 1962). Subsequently it has been found possible to extract soluble H-2 antigens from mouse cells by washing in hypotonic solution (Haughton, 1964; Davies, 1966) or with the enzyme ficin (Nathenson and Davies, 1966). The antigenic material seems to be a glycoprotein (Davies, 1967). This approach has been applied to the mouse thymus leukemia antigen (Davies, Boyse, Old, and Stockert, 1967) and may prove useful for other surface antigens.

9. Antigen studies on hybrid cells

Studies of hybrid species and cells in tissue culture have shown that

the antigens of each parent can exist on the same cell. For example, the red blood cells of a dove hybrid carried the antigens of each parent as well as an additional antigen (Irwin, 1932). Studies of hybrid cells in tissue culture began with the work of Barski, Sorieul, and Cornefert (1960). Two different strains of mouse cell were grown together and a hybrid, differing from each of the parents in morphology and degree of malignancy, eventually appeared. Hybrid cells have also been produced by growing cell lines which lack certain enzymes in selective medium (Littlefield, 1964) or by growing mixed cultures at 29^{o}C (Scaletta and Ephrussi, 1965). Viable hybrids have been obtained not only between various strains of mouse cells but also between rat and mouse cells (Ephrussi and Weiss, 1965) and mouse and hamster cells (Davidson, Ephrussi, and Yamamoto, 1966). Antigen studies on these cells reported to date have concerned only hybrids between strains of mouse cell. Unlike heterokaryons produced with virus which can be studied immediately after fusion, these hybrid cells have been grown for many generations to obtain a sufficiently large and homogeneous population for study.

Gershon and Sachs (1963) studied a hybrid between mouse L cells and a polyoma-induced mammary tumour. Growth of the hybrid cells in mice suggested that the antigens of each parent and the polyoma antigen were still expressed, although the data from a small number of experi-

ments are not convincing. The studies of Defendi, Ephrussi, and Koprowski (1964) and Defendi, Ephrussi, Koprowski, and Yoshida (1967) suggested the persistence of polyoma transplantation antigen in mouse hybrid cells. Several H-2 antigens can co-exist on viable mouse hybrid cells produced in vitro (Spencer, Hauschka, Amos, and Ephrussi, 1964). All these studies utilized the growth of hybrid cells in vivo and the elution of absorbed antiserum in vitro to identify antigens rather than direct labelling of hybrid cells.

The presence of specific hybrid antigens remains uncertain, although recent work has suggested that the genes of each parent may contribute to enzyme synthesis in a rat-mouse hybrid (Weiss and Ephrussi, 1966a, 1966b). Hybrid antigen molecules can occur in humans as shown by the AB blood group substance (Morgan and Watkins, 1956), and individual antigen molecules bearing determinants contributed by each parent have been found in hybrid clones of paramecium (Finger, Onorato, Heller, and Wilcox, 1966).

10. Mechanism of virus-induced cell fusion

Harris et al. (1966) have reviewed the history of cell fusion and observations on multinucleate cells. The actual mechanism of cell fusion is poorly understood. Sendai virus agglutinates cells at 4^{o}C, but fusion takes place only when the cells are warmed (Okada, 1962). Even cells

which ordinarily grow at 18°C will only fuse at 37°C with Newcastle disease virus (Melendez, Spalatin, and Hanson, 1965). Calcium ions are essential, apparently, for actual cell fusion rather than virus adsorption (Okada and Murayama, 1966), and cells must contribute energy for fusion to occur (Okada, Murayama, and Yamada, 1966).

This process indicates differences in cell surface structure. Okada and Tadokoro (1963) reported that established cell lines fused well with Sendai virus, short-term cultures poorly, and leucocytes and lymphocytes did not fuse at all. Apparently there is some surface alteration during prolonged culture which increases the susceptibility of cells to viral fusion. The work of Harris et al. (1966) shows that cells such as lymphocytes can be fused with established cell lines. Kohn (1965) studied cell fusion produced by Newcastle disease virus. The ability to fuse cells was associated with phospholipids of the viral coat and was separate from viral neuraminidase, haemagglutinin, haemolysin, and infectivity.

Under the electron microscope the process of cell fusion appears to be similar for different cell types (Schneeberger and Harris, 1966). Cytoplasmic bridges formed between cells, but no virus was seen at the site of these bridges. Viral induction of microvilli did not seem to be important, although those cells which possessed microvilli (Ehrlich cells, HeLa cells, rabbit macrophages) fused most readily. Hen erythrocyte fusion

with HeLa cells was found to involve the following steps: lysis of erythrocytes with loss of cytoplasm, agglutination of the ghosts to HeLa cells, flow of HeLa cytoplasm into the ghost at 37°C, entry of the erythrocyte nucleus into the HeLa cell, and establishment of continuity of the erythrocyte membrane with the HeLa cell surface.

11. Surface properties of cells used in fusion experiments

For fusion experiments the HeLa-Ehrlich combination was the obvious choice for initial studies, since these heterokaryons were the first to be formed and have already been studied in some detail (Harris and Watkins, 1965; Harris et al., 1965; Harris et al., 1966). There is the additional advantage that HeLa and Ehrlich nuclei are easily distinguished. It would be interesting to compare these heterokaryons with HeLa - L heterokaryons, since L cells grow well in vitro while Ehrlich cells do not. Various other cell combinations were chosen for special reasons. Freshly isolated embryo cells are diploid and might give viable hybrids after fusion with established cell lines. Macrophages have specialized surface characteristics. Lymphocytes are immunologically competent cells. Erythrocytes are particularly interesting because no cytoplasm enters heterokaryons and because the dormant nucleus starts to synthesize DNA and RNA.

(i) HeLa cells

HeLa cells are a malignant epithelial cell line derived from

a human cervical carcinoma (Scherer, Syverton, and Gey, 1953). They carry human species antigens (Brand and Syverton, 1960; Coombs et al., 1961 a) and H blood-group antigen (Kelus, Gurner, and Coombs, 1959). They do not carry the Forssman antigen (Coombs et al., 1961 b) but apparently have a specific antigen which is not present on normal human cells (McKenna, Sanderson, Davis, and Blakemore, 1966). At least 8 groups of antigens are shared by HeLa cells and human erythrocytes (Brand and Chiu, 1966 a). Antiserum to HeLa cells has been shown to cross-react weakly with L cells by complement-fixation (Kite and Merchant, 1961) but not with L cells or Ehrlich ascites cells by agar diffusion (Boyle, Davies, and Haughton, 1963).

(ii) Ehrlich cells

Ehrlich cells have been carried as an ascitic tumour in the peritoneal cavity of non-inbred mice for many years. It has been suggested that this type of non-specific tumour has a decreased concentration of H-2 isoantigens (Moller, 1964). This tumour apparently bears some antigens not found in normal mice, since growth can be prevented by immunizing animals with living irradiated cells (Donaldson and Mitchell, 1959) or by serial tapping of tumour-bearing mice (Apffel, Arnason, Twinam, and Harris, 1966). These cells share a number of antigens with mouse L strain fibroblasts (Boyle et al., 1963). Ehrlich cells seem to share more antigens with embryonic mouse erythroblasts than

with adult mouse erythrocytes (Furusawa, Adachi, and Asayama, 1965; Furusawa, Kotani, Takeuchi, and Asayama, 1965). On the surface of Ehrlich ascites cells there is a considerable amount of sialic acid (Wallach and Eylar, 1961; Gasic and Berwick, 1963) and glycoprotein (Eylar and Cook, 1965). The Ehrlich ascites cells used in this laboratory do not stick to glass or grow in vitro.

(iii) L cells

Mouse L strain fibroblasts were derived from the subcutaneous tissue of C3H mice and the cells were treated with methylcholanthrene in vitro (Earle, 1943). They carry mouse species antigens (Coombs, Daniel, Gurner, and Kelus, 1961 c) and the H-2 antigens of the C3H mice of origin (Gangal, Merchant, and Shreffler, 1966). At least one group of antigens is shared with human cells as shown by immuno-diffusion (Brand and Chiu, 1966 b). L cells come from a Forssman-positive species but have lost this antigen in culture (Coombs et al., 1961 b). Tumours induced by methylcholanthrene have a new antigen as shown by transplantation studies (Foley, 1953; Prehn and Main, 1957; Klein, Sjögren, Klein, and Hellstrom, 1960) and with fluorescent antibody (Pilch and Riggins, 1966). This antigen is probably present on L cells.

(iv) Hen and chick erythrocytes

Fowl erythrocytes carry species antigens but there are differ-

ences in blood group antigens first recognized by Landsteiner and Miller (1924). The blood groups have been named A and B, but the situation is complicated since there is complete lack of antigen dominance and many antigen combinations can occur (Briles, McGibbon, and Irwin, 1950). The species antigen may be protein as has been suggested for the species antigens of guinea pig erythrocytes (Brand, 1963), while the blood group substances may be mucopolysaccharide like those of human erythrocytes. The hen is a Forssman-positive species.

Harris (unpublished observations) has shown that chick embryo erythrocytes fuse more readily with HeLa cells than adult erythrocytes, and it was planned to carry out antigen studies on each type of heterokaryon. There are differences in the antigens on embryo and adult erythrocytes as shown by haemagglutination studies (Levi and Schectman, 1954, 1964). This has been demonstrated with fluorescent antibody by D'Amelio (1966), who also showed that adult and early chick embryo erythrocytes differ with respect to methanol fixation and tryptic digestion. In comparison with mammalian erythrocytes, nucleated erythrocytes show increased resistance to membrane stretching and haemolysis with water and greater membrane elasticity can be demonstrated with a needle (Jacobs, 1962). It has been known for many years that

nucleated erythrocytes have an equatorial band about the periphery (Meves, 1911) and a marginal band of tubular structures encircling some types of nucleated erythrocyte has been seen just beneath the plasma membrane in electron micrographs (Fawcett, 1959).

(v) Macrophages

These cells have the ability to adsorb antibody from solution. This type of reaction was shown by Boyden and Sorkin (1960) with rabbit spleen cells, although the demonstration of antibody up-take by normal tissue was first shown many years before (Dale, 1912-13). Macrophages sensitized with anti-erythrocyte antibody will adsorb erythrocytes (Boyden, 1964; Jonas, Gurner, Nelson, and Coombs, 1965), and sensitized erythrocytes are avidly adsorbed by macrophages (Watkins, 1965; Berken and Benacerraf, 1966). The possible complications of this reaction for antigen studies on heterokaryons will be discussed under Results.

The nature of the macrophage receptors for this 'cytophilic antibody' is not well understood, although they are affected by oxidizing agents and reagents which act on free sulphydryl groups (Howard and Benacerraf, 1966) and may have phospholipid or phospholipoprotein as an essential component (Davey and Asherson, 1967). It would be of interest to see if this property is still expressed after the fusion of

macrophages with established cell lines.

(vi) Other cells

Lymphocytes carry species antigens. They are capable of initiating immunological reactions (Gowans, MacGregor, Cowen, and Ford, 1962) and can destroy cells in tissue culture as previously discussed. The manner in which the lymphocyte recognizes and destroys foreign cells is not well understood.

The ability of embryo cells to reaggregate and the presence of external coatings about some embryo cells (Lesseps, 1967) have already been mentioned.

12. Summary of objectives

The preceding review describes some of the many complex reactions in which the cell surface participates and illustrates how poorly the structure and function of the cell surface is understood, particularly in molecular terms. The effect of cell fusion on surface antigens and surface properties is difficult to predict, and it is evident that an explanation of results may be even more difficult.

The objectives of this study may be summarized as follows:

(i) To obtain information about the behaviour and metabolism of the cell surface and cell surface antigens from a study of their behaviour in heterokaryons.

(ii) To use the labelling of surface antigens as a means of identifying hybrid cells with certainty, of screening populations of fused cells for division, and perhaps as a method of isolating a hybrid cell line if hybrid growth should occur.

(iii) To see if the presence or absence of surface antigens could be used to indicate metabolic activity and protein synthesis by each component of a heterokaryon.

MATERIALS AND METHODS

1. Cells

(i) Established cell lines

HeLa cells were maintained as a monolayer culture and have been grown in this laboratory for some years (Harris and Watts, 1962). They were not obtained from a clone.

Mouse L strain fibroblasts were obtained from Dr. R. J. Berry of the Churchill Hospital, Oxford. For later experiments L-929 cells were obtained from Flow Laboratories, Irvine, Scotland. These cells were grown as a monolayer.

(ii) Transplanted tumour

A tetraploid line of Ehrlich ascites cells was maintained by weekly serial passage in non-inbred Swiss mice.

(iii) Primary cell cultures

Embryos were obtained from 13-day-old fertile hens' eggs, and from pregnant mice and hamsters after 12 days of gestation. After decapitation the embryos were passed through a mincer and then suspended in a 0.05% solution of trypsin (Difco) in Hanks' solution (Hanks, 1948). After incubation for 10 minutes at 37°C and frequent shaking, tissue fragments were allowed to settle and suspended cells removed with a Pasteur pipette. Calf serum was added to inactivate the trypsin and the cells counted in a Neubauer counting chamber (Hawksley, London). About 5×10^6 cells were seeded into medical flat bottles in 10 ml. of medium.

(iv) Macrophages

Mouse peritoneal macrophages were obtained by injection of 5 ml. of medium 199 (see next section) containing 20 units of heparin (Evans Medical Ltd., Liverpool) per ml. into the peritoneal cavity of freshly killed adult mice and aspiration of the washings.

Rabbit macrophages were obtained from the peritoneal cavity by the technique of Mackaness (1952) and were kindly supplied by Dr. A. M. Woodin. About 50 ml. of heavy liquid paraffin (Harrington Bros., London) were injected under sterile conditions into the peritoneal cavity of an adult rabbit and the resulting exudate washed out 4 to 6 days later with saline.

(v) Lymphocytes

These were obtained from the thoracic duct of non-inbred rats and were kindly supplied by Dr. P. J. McCullagh and Mr. J. C. Howard. The cell population is virtually pure and contains about 95% small lymphocytes and 5% large lymphocytes.

(vi) Hen erythrocytes

The method of obtaining hen erythrocytes and removing leucocytes has been described by Harris et al. (1966). Venous blood was placed in a solution of heparinized saline and the leucocytes removed by repeated centrifugation in capillary tubes.

(vii) Chick embryo erythrocytes

These were obtained from the allantoic vessels of 13- to 14-day-old embryos. The shell over the air sac was removed and the chorio-allantoic membrane stripped off. Several vessels were cut with scissors

and the red cells removed with allantoic fluid. Care was taken not to damage the yolk sac.

2. Solutions and Media

(i) Phosphate buffered saline (PBS)

This buffer, described by Dulbecco and Vogt (1954), was prepared from tablets (Oxoid) dissolved in double distilled water and sterilised by autoclaving. The pH is 7.3.

(ii) 6.8 buffer

Tablets (G. T. Gurr, London) dissolved in distilled water were used to prepare this phosphate buffer of pH 6.8.

(iii) Hanks' solution

Hanks' solution is a balanced salt solution of pH 7.4. A powder (Oxoid) was used to prepare stock solutions A and B. 10 ml. of each solution were mixed with 80 ml. of double distilled water and sodium bicarbonate added to give the correct pH.

(iv) Hanks' solution without glucose.

The required salts were dissolved in double distilled water without glucose or dextrose. This solution was used as the suspending medium in cell fusion experiments since the high concentrations of cells cause marked changes of pH in solutions containing glucose and the process of cell fusion is pH sensitive.

(v) Medium 199 (Morgan, Morton and Parker, 1950)

This is a complex amino acid-containing medium in balanced salt solution. It was obtained in concentrated form from Glaxo Laboratories,

Greenford, and was diluted ten times in sterile double distilled water before use. In diluted form it contains 200 units of penicillin and 100 ug of streptomycin per ml.

(vi) Culture medium

80 ml. of medium 199 was mixed with 10 ml. of a 5% solution of tryptose broth (Difco) and 10 ml. of serum for each 100 ml. of culture medium. Calf serum was obtained every two weeks from Burroughs Wellcome and was stored at 4°C. Foetal calf serum from Flow Laboratories, Irvine, Scotland, was kept at -20°C. Rabbit serum was obtained from laboratory animals as needed and inactivated at 56°C for 30 minutes immediately after separation. It was stored at -20°C. All sera were Sietz filtered before use and the medium stored at 4°C. Kanamycin (Bayer) to give a final concentration of 50 ug/ml. and mycostatin (Squibb) 40 ug/ml. were added to the medium.

3. Virus

(i) Growth

The details of virus growth have been fully described by Harris et al. (1966). Sendai virus was grown in the allantoic cavity of 11-day-old fertile hens' eggs. After 3 days at 37°C and about 12 hours at 4°C the allantoic fluid was removed and the virus spun out at 30,000 g with a Spinco Model L-50 ultracentrifuge.

(ii) Titration

Haemagglutination with sheep erythrocytes was carried out with allantoic fluid from infected eggs and with suspensions of concentrated

virus. Virus was diluted in Hanks' solution without glucose and titration performed in a Salk-pattern haemagglutination tray. The smallest amount of virus which produced complete haemagglutination after 2 hours at room temperature was defined as one haemagglutination unit (HAU).

(iii) Inactivation

A concentrated virus suspension in a watch glass was exposed to ultraviolet light at an intensity of 3,000 ergs/cm^2/sec for 3 minutes. Infectivity was decreased by more than 10^6 while the HAU titre and fusion capacity were unchanged. Diluted virus was given a similar dose of irradiation before being used for cell fusion.

(iv) Storage

Stock infectious virus in allantoic fluid, concentrated inactivated virus, and various dilutions of inactivated virus in Hanks' solution without glucose were rapidly frozen with solid CO_2 in ethyl alcohol and stored at $-70^{\circ}C$.

4. Cell Fusion

The technique of cell fusion was based on that of Okada (1962) and has been described by Harris et al. (1966). HeLa cells, L cells or primary fibroblast cultureswere trypsinized and washed once in PBS with added calf serum before use. Ehrlich ascites cells from the mouse peritoneal cavity, rat lymphocytes, hen and chick erythrocytes, and mouse and rabbit macrophages were washed twice in PBS and usually used within an hour of withdrawal. The

virus dose and cell numbers for each fusion will be given under Results. Each cell type was suspended in 1 ml. of Hanks' solution without glucose and placed in a chilled T-tube. A suspension of inactivated virus, also in Hanks' solution without glucose, was then added. The tube was kept at 4°C for 15 minutes to allow cell clumping to occur and then shaken in a water bath at 37°C for 5 minutes. Culture medium was then added to the T-tube and the fused cells placed in Petri dishes or medical flat bottles.

5. Growth of Cells

Established cell lines and primary fibroblast cultures were grown as monolayers in 100 ml. medical flat bottles in 10 ml. of culture medium. Cells were harvested for subculture every 3 or 4 days using 0.1% trypsin in PBS. In some experiments HeLa cells were harvested with 0.02% EDTA (ethylenediamine tetra acetic acid, British Drug Houses) in PBS. Heterokaryons and cultures of single cells for antigen analysis were grown on circular glass coverslips 11 mm. in diameter (Chance) in 60 mm. plastic Petri dishes (Esco Plastics, London) containing 5 ml. of culture medium. The dishes were incubated at 37°C in a gas mixture of 5% CO_2 in air. Coverslips bearing heterokaryons were transferred to fresh medium 24 hours after fusion. HeLa-lymphocyte and HeLa-erythrocyte heterokaryons were placed in fresh medium 12 hours after fusion. For all heterokaryon cultures, medium was generally changed every 48 hours.

6. Examination of Cells

Coverslips from Petri dishes were rinsed in PBS, fixed in methanol, washed in pH 6.8 buffer, and stained in 10% Giemsa stain (G. T. Gurr, London). Time of staining varied from 5 minutes for cultures with few cells to 20 minutes for confluent monolayers. For some experiments living cells were examined by light or phase contrast microscopy through the wall of the Petri dish. For counting cells, at least 20 high power fields were examined per coverslip. Fields were selected at 1 mm. intervals with the stage scale of the microscope along paths at right angle to each other and the full diameter of the coverslip was spanned. The number of cells per coverslip was:

$$\frac{\text{cells counted in 20 fields}}{20} \times 930$$

For sparse cell populations more fields were examined so that at least 100 cells were counted.

7. Autoradiography

Radioactive precursors used were uridine-5[^{3}H], specific activity 13 C/mM, thymidine-6[^{3}H], specific activity 5 C/mM and DL-leucine-4,5[^{3}H], specific activity 5 C/mM. These were obtained from the Radiochemical Centre, Amersham. All agents were used at a concentration of 10 uc/ml. in Hanks' solution containing 10% calf serum. Length of exposure of coverslip cultures to the precursor was 30 minutes for uridine, 60 minutes for thymidine, and 2 hours for leucine and all incubation was at 37^{o}C. Coverslips were then rinsed in three changes of PBS and

Table 1

Immunization Schedule for Anti-cell Sera Produced in Rabbits

Cells	Number of cells per injection	Number of weekly injections
HeLa	10^7	4
L	2×10^6	6
Ehrlich ascites	10^8	4
Rat lymphocytes	10^9	4
Hen erythrocytes	10^9	3
Hamster erythrocytes	1 ml 50% suspension	3
Hamster embryo	3×10^6 5×10^7	2 2
Chick embryo	10^7	3

Table 2

Immunization Schedule for Anti-cell Sera Produced in Guinea Pigs

Cells	Number of cells per injection	Number of weekly injections
HeLa	5×10^6	4
L	5×10^6	4
Ehrlich ascites	10^7	3
Hen erythrocytes	5×10^8	4
Sheep erythrocytes	1 ml 20% suspension	4

fixed for 30 minutes in methanol at 4°C. Fixed preparations were extracted with 0.3 N trichloracetic acid at 4°C for 30 minutes. For experiments combining autoradiography with mixed haemadsorption it was found necessary to leave out trichloracetic acid extraction because of the destruction of erythrocytes. Coverslips were then left for 2 hours in distilled water at room temperature. Autoradiographs were made by the stripping film technique (Pelc, 1947) using Kodak AR 10 film. Exposure was usually 7 days. After development of the film, cells were stained through the emulsion with Leishman's stain (E. Gurr, London).

8. Antisera

(i) Rabbit

Rabbits were given weekly intravenous injections of whole cells and bled out one week after the last injection. HeLa and L cells were grown in medium containing rabbit serum for many generations before injection to avoid the formation of antibody to adsorbed serum proteins (Hamburger, Pious and Mills, 1963). Ehrlich ascites cells, hen erythrocytes, and rat lymphocytes were obtained fresh and all cells were washed in PBS before use. Table I shows the cell numbers and injections.

For some antisera rabbits were injected with cells in complete Freund's adjuvant (Difco) as described by Freund (1947). Injections were given twice weekly in 4 separate intramuscular sites and the animals bled one week after the last injection. For antiserum to

Ehrlich ascites cells the rabbit was first given 2 x 10^6 cells in two intravenous injections and for each of the following sets of injections given in 2 weeks, 10^9 cells were emulsified with adjuvant. Antisera to chick embryo extract and chick embryo erythrocytes were kindly supplied by Dr. R. R. Wagner. For the chick embryo extract, 11-day-old embryos were minced, ground, and frozen and thawed 4 times in PBS. The supernatant was mixed with adjuvant and the animal injected twice weekly for 6 weeks. One ml. of packed chick embryo erythrocytes in 1 ml. of adjuvant was injected twice weekly for 3 weeks.

(ii) Guinea pig

Intraperitoneal injections of whole cells were carried out weekly and the animals bled out one week after the last injection. The number of cells injected is shown in Table 2.

All rabbit and guinea pig antisera were inactivated at 56°C for 30 minutes immediately after separation and kept at -70°C. Some sera were preserved with 30% glycerol (v/v) and sodium azide to 0.1% (w/v) and stored at 4°C.

(iii) Commercial antisera

These will be described under mixed haemadsorption.

9. Antigen Labelling of Tissue Culture Cells

(i) Mixed agglutination

Monolayers of cells growing on coverslips were rinsed in PBS and placed in the cups of a Salk-pattern haemagglutination tray to which had been added antiserum dilutions in Hanks' solution. After incubation

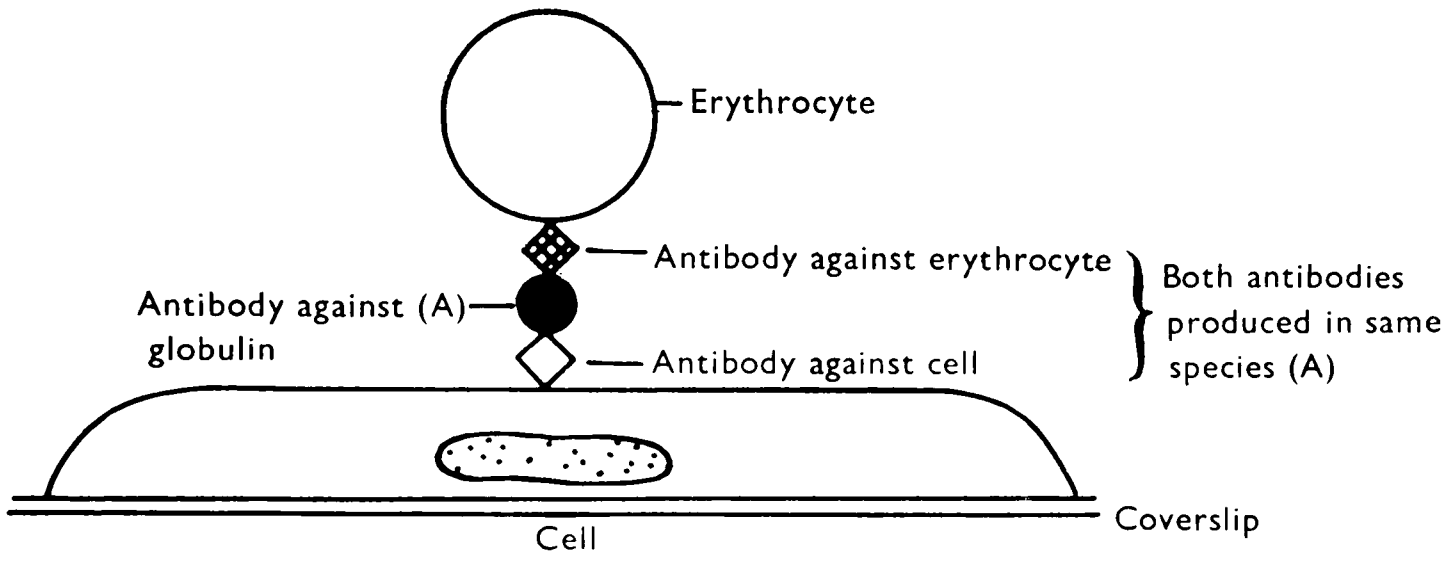

Figure 1. Diagrammatic representation of the principles of the mixed haemadsorption (mixed antiglobulin) technique applied to tissue culture cells.

for 30 minutes at room temperature, the coverslips were rinsed in PBS and placed in another cup containing a 0.5% suspension in Hanks' solution of washed erythrocytes of the same species as the tissue culture cells. After allowing one hour for the red cells to settle the coverslips were rinsed 10 times in PBS, fixed in methanol and stained with Giemsa.

(ii) Immune adherence

Antiserum dilutions in Hanks' solution were placed in the cups of a Salk-pattern haemagglutination tray with 1/20 preserved guinea pig complement (Burroughs Wellcome). A coverslip culture was rinsed in PBS and incubated in antiserum for 30 minutes at 37°C. After another rinse in PBS the coverslip was placed in a cup containing a 0.5% suspension of fresh human group O erythrocytes in Hanks' solution. These were allowed to settle for one hour at 37°C. The coverslip was then washed 10 times in PBS and fixed and stained as usual.

(iii) Mixed haemadsorption (mixed antiglobulin technique)

The method has been described by Watkins and Grace (1967) and is based on that of Espmark and Fagraeus (1965). The principles are illustrated in Fig. 1. Sheep erythrocytes (Burroughs Wellcome) were delivered weekly and used within one week. A 4% suspension in Hanks' solution was sensitised with 0.025 ml. of rabbit antiserum to sheep erythrocytes and doubly sensitised with 0.2 ml. of sheep antiserum to rabbit serum (both antisera from Burroughs Wellcome). Washed doubly sensitised erythrocytes at a concentration of 4% were suspended in Hanks' solution with added kanamycin. For later experiments

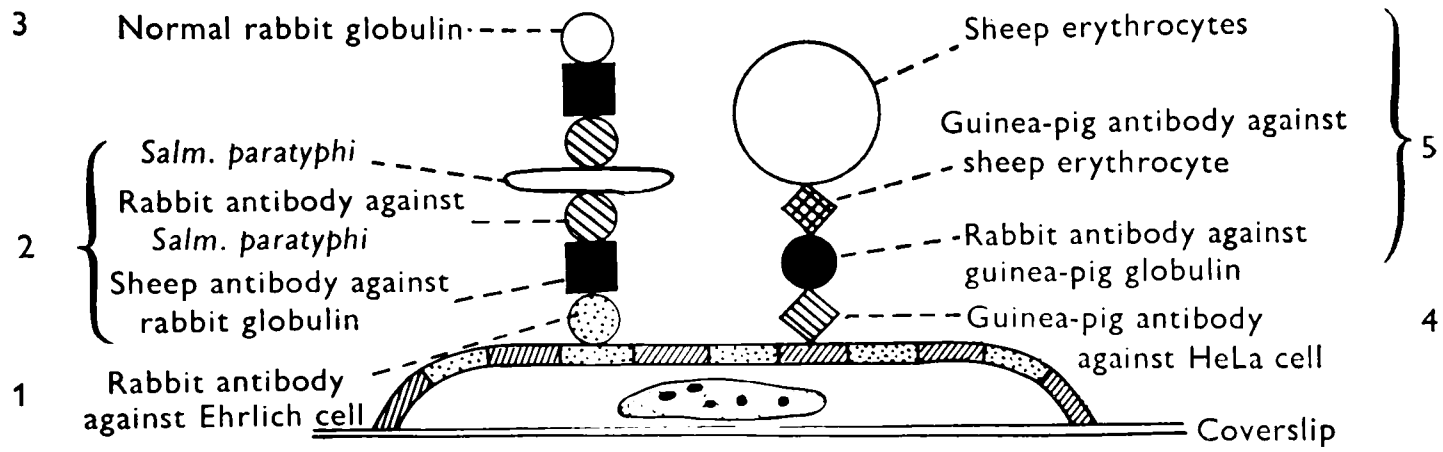

Figure 2. Principles of the double labelling technique applied in this case to a HeLa-Ehrlich heterosynkaryon.

doubly sensitised erythrocytes were suspended in culture medium. This resulted in better preservation of tissue culture cells without affecting haemadsorption. Coverslip cultures were incubated in antiserum dilutions in Hanks' solution for 15 minutes at room temperature, rinsed in PBS, and 0.5% doubly sensitised erythrocytes allowed to settle for one hour. The coverslips were rinsed 10 times in 3 changes of PBS and fixed and stained as usual.

(iv) Elimination of viral haemadsorption

Advantage was taken of the fact that Sendai virus, like other myxoviruses, will elute from erythrocytes to which it has adsorbed. After carrying out mixed haemadsorption on heterokaryon cultures and rinsing in PBS, the coverslips were incubated for another hour in culture medium to allow erythrocytes adherent to virus to elute. If there was no viral haemadsorption on control cultures several days after fusion, this post-incubation step was eliminated.

(v) Double labelling

This has also been described in detail elsewhere (Watkins and Grace, 1967) and is illustrated in Fig. 2. Erythrocytes mark HeLa antigen and bacteria mark Ehrlich antigen. An alcohol-killed suspension of Salmonella paratyphi A and rabbit antiserum to paratyphi AO were obtained from Burroughs Wellcome. Rabbit antiserum to guinea pig serum was also from Burroughs Wellcome. For later experiments rabbit antiserum to guinea pig globulin, kindly supplied by Professor R. R. Coombs, was used at a concentration of 1/100 for double sensitisation of sheep erythrocytes.

The most confusing step in this procedure may be the use of normal serum. The doubly sensitised bacteria have sheep-v-rabbit globulin as the outer layer. Some of these antibody molecules will be attached to rabbit globulin on the surface of Ehrlich cells or heterokaryons but some will also be free on the outer surface of the bacteria. The doubly sensitised erythrocytes have rabbit globulin as the outer layer and this would attach to the free sheep-v-rabbit globulin groups on the bacteria and give false positive double labelling unless normal rabbit serum were used to prevent this complication.

Control experiments were carried out to validate this technique (Watkins and Grace, 1967) and showed that:

1. The antisera were cell-specific.
2. The markers did not adhere to each other.
3. Attachment of the first antibody to the cell surface did not prevent attachment of the second antibody to adjacent antigens of the other species.
4. Spatial competition between the two markers at the surface of heterokaryons did not prevent labelling. It is apparent that very heavy labelling with bacteria could prevent erythrocyte labelling on heterokaryons.

(vi) Specificity of antisera

Cross-reaction of antisera on heterologous cells in mixed haemadsorption experiments was decreased by absorbing 1 ml. of antiserum diluted 1/10 with 10^8 cells for one hour at $37^{\circ}C$ in a shaker and for 2 hours at $4^{\circ}C$. Cross-reaction was abolished by diluting the absorbed

antiserum to a point at which a strong positive reaction was obtained on the cells to which the antiserum was produced and no reaction on the other cell type to be used in a particular fusion experiment. For studies on HeLa-Ehrlich heterokaryons, anti-HeLa serum was absorbed with Ehrlich cells and anti-Ehrlich serum with HeLa cells. The same procedure and the same doses of absorbing cells were used for HeLa-L heterokaryons. The method of in vivo absorption of anti-L serum to obtain specific labelling on Ehrlich-L heterokaryons and similar antiserum absorption for hamster fibroblast-L cell heterokaryons will be given under Results. Antisera to fowl erythrocytes and fibroblasts showed little cross-reaction on established cell lines even at low dilutions.

For HeLa-Ehrlich heterokaryons, anti-HeLa serum was used at a dilution of 10^{-3} and anti-Ehrlich serum at a dilution of 5×10^{-3}. The same dilutions were used for HeLa-L heterokaryons. The dilutions of antisera for studies on other heterokaryons will be given under Results. Dilutions of antisera which gave specific labelling were stored in 1 ml. quantities at $-70^{\circ}C$.

10. Cytolysis

(i) In suspension

Antisera were first titrated for cytolytic antibody using suspensions of cells because of the sharp end-point which could be obtained. The method was a modification of that described by Spooner, Bowden and Carpenter (1965). Dilutions of unabsorbed antiserum were prepared in Hanks' solution. Trypsinised HeLa or L cells or freshly aspirated

Ehrlich ascites cells washed in Hanks' solution were prepared as suspensions with 2 x 10^5 cells per ml. Preserved guinea pig complement (Burroughs Wellcome) at a dilution of 1/10 was mixed with an equal volume of cell suspension. One drop (0.025 ml.) of antiserum dilution and one drop of cell suspension in complement were then mixed on a perspex plate. The total number of cells added was about 2,500. The antiserum concentration has been expressed as the final dilution with cells and complement. The plates were incubated in a moist atmosphere at 37°C for 90 minutes. The drops were then covered with glass coverslips and examined under phase contrast. Lysed cells were counted directly. The titre is expressed as that antiserum dilution at which 50% of cells are lysed. Determination of cell viability with trypan blue gave exactly the same results but took a longer time and was therefore not used.

(ii) <u>On monolayers</u>

Antiserum dilutions in Hanks' solution were mixed with an equal volume of 1/20 preserved guinea pig complement in cups of a Salk-pattern haemagglutination tray. Coverslips on which were growing unfused cells or heterokaryons were rinsed in PBS and incubated in the antiserum for 2 hours at 37°C. They were then fixed in methanol and stained with Giemsa (G. T. Gurr Ltd.). For some experiments heterokaryons were incubated for 24 hours in antiserum dilutions in medium without complement. Fresh guinea pig serum diluted 1 in 20 was used as the source of complement for some cytolysis experiments.

11. Radiation of Cells

(i) Cobalt 60

Freshly trypsinised monolayer cells were spun to the bottom of a centrifuge tube. Gamma-radiation from a cobalt 60 source was given through the glass at a rate of 1,000 r per minute.

(ii) Ultraviolet irradiation

Irradiation was carried out with a Philips 15-W 18-inch germicidal tube, type 'T.U.V.', as described by Harris (1967). 10^7 Ehrlich ascites cells were suspended in 5 ml. of PBS and placed in a 60 mm. Petri dish and the dish was rotated during irradiation. The intensity of radiation at the fluid surface was about 10,000 ergs/mm^2 for a 3-minute period and doses ranged from 5,000 to 50,000 ergs/mm^2.

12. Cloning of Cells with Feeder Layers

This was based on the work of Puck and Marcus (1955, 1956). Tissue culture cells to be cloned, usually about 10^4, were mixed with 5×10^5 HeLa cells given 6,000 or 8,000 r of gamma-radiation. The cells were placed in a 60 mm. Petri dish, containing coverslips if required, in 5 ml. of culture medium. Colonies were counted 7 to 10 days after plating of cells.

13. Antibody Persistence on Cultured Cells

The method of labelling cells with dilute antiserum, of washing these cells, of checking for and preventing antibody transfer, and of following the persistence of antibody on the cell surface will be discussed under Results.

14. Thymidine Labelling of Cells

Cells labelled with [^{3}H]-thymidine were required for some studies on the persistence of antibody on the cell surface. HeLa cells growing on a monolayer 24 hours after plating were exposed to 50 uc of [^{3}H]-thymidine in 10 ml. of culture medium over a period of 24 hours. The medium was then poured off and the monolayer cells washed once with PBS. Trypsinised cells were washed 3 times in an excess of PBS, and the wash fluid checked for residual radioactivity in a scintillation counter. The fluid from the third wash had a radioactive level the same as background.

15. Other Chemicals and Reagents

(i) Neuraminidase (N-acetyl-neuraminate glycohydrolase) with an activity of 500 units/ml. was obtained from British Drug Houses, Poole.

(ii) Actinomycin was a gift from Merck, Sharp and Dohme, Inc.

(iii) Puromycin-2-HCl from the American Cyanimid Company, Pearl River, New York was supplied by Professor Abraham.

(iv) Active leukocidin, prepared from the two components (Woodin, 1960) was kindly supplied by Dr. A. M. Woodin.

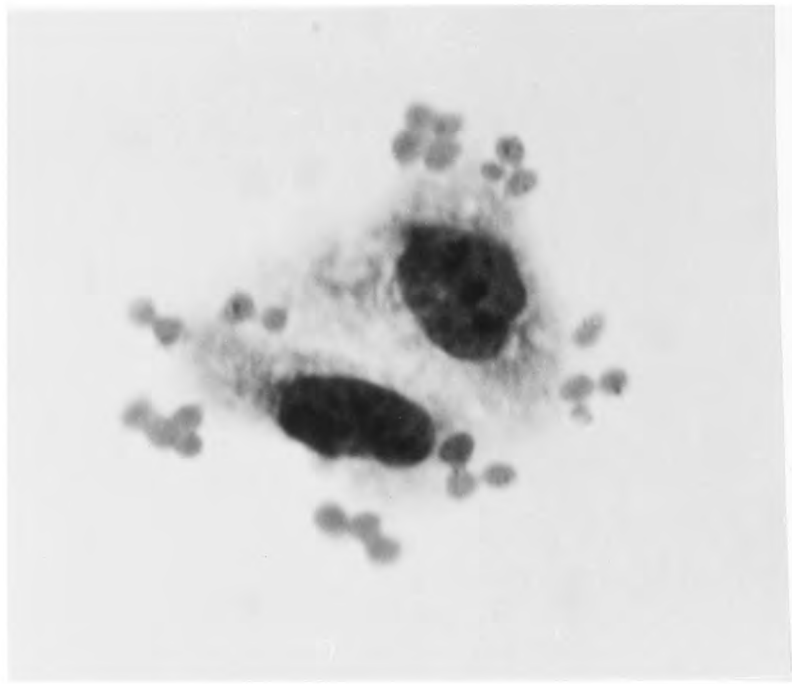

Figure 3. Mixed agglutination of human erythrocytes to HeLa cells using rabbit anti-HeLa serum at a dilution of 1/10. x 720.

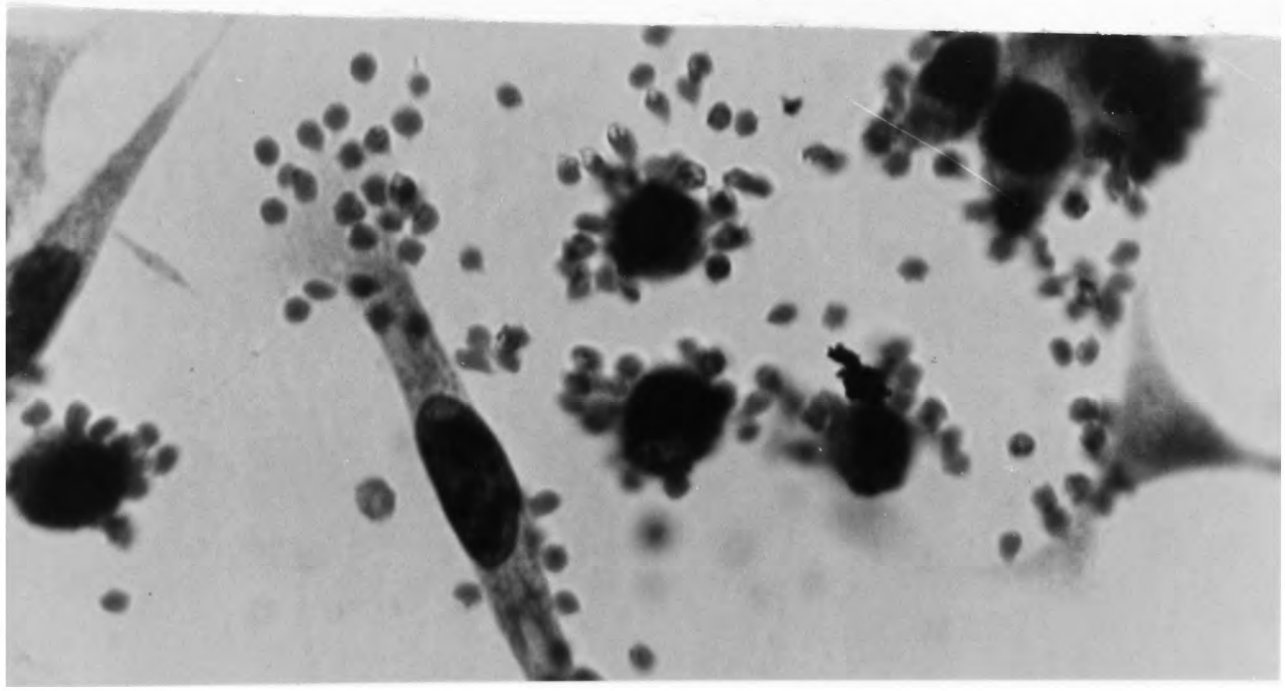

Figure 4. Mixed agglutination of mouse erythrocytes to mouse L strain fibroblasts using rabbit anti-L serum at a dilution of 1/10. Some of the L cells have rounded up because of the effect of strong antiserum. x 720.

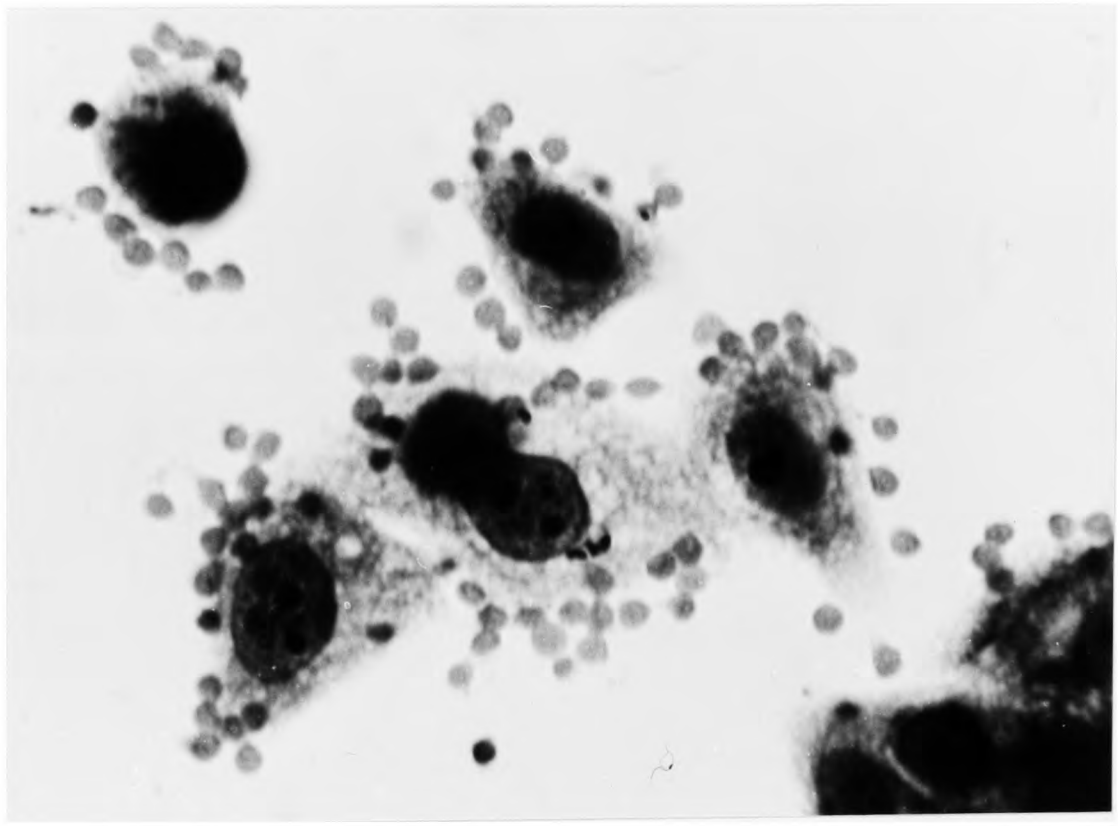

Figure 5. Immune adherence of human group O erythrocytes to HeLa cells with rabbit anti-HeLa serum at a dilution of 1/40. Many HeLa cells came off the glass after treatment with antiserum and complement. x 720.

RESULTS

1. Detection of Surface Antigens

(i) Mixed agglutination

Good macroscopic haemadsorption was obtained on monolayers of HeLa cells with human group O erythrocytes and rabbit anti-HeLa serum at a concentration of 1/2 or 1/10. Similarly, haemadsorption was obtained on L-cell monolayers with mouse erythrocytes and rabbit anti-L serum at a dilution of 1/2 or 1/10. However, when coverslips were rinsed in PBS and fixed and stained, microscopic haemadsorption tended to be patchy as shown in Figs. 3 and 4. Moreover, isolated cells were often completely unlabelled.

(ii) Immune adherence

Rabbit anti-HeLa serum was haemolytic for human erythrocytes to a dilution of 1/64 in the presence of complement. Thus it was necessary to sensitise tissue culture cells with antibody and complement in one step and allow human erythrocytes to settle in a second. With 1/40 rabbit anti-HeLa serum,a concentration at which mixed agglutination was not obtained, quite good immune adherence occurred as shown in Fig. 5. However, the labelling was patchy and often HeLa cells were not completely surrounded by erythrocytes. No immune adherence was obtained with anti-HeLa serum at a dilution above 1/100.

(iii) Mixed haemadsorption

The lack of sensitivity, the failure to obtain reliable haemadsorption on all monolayer cells, and the possibility of damaging cells

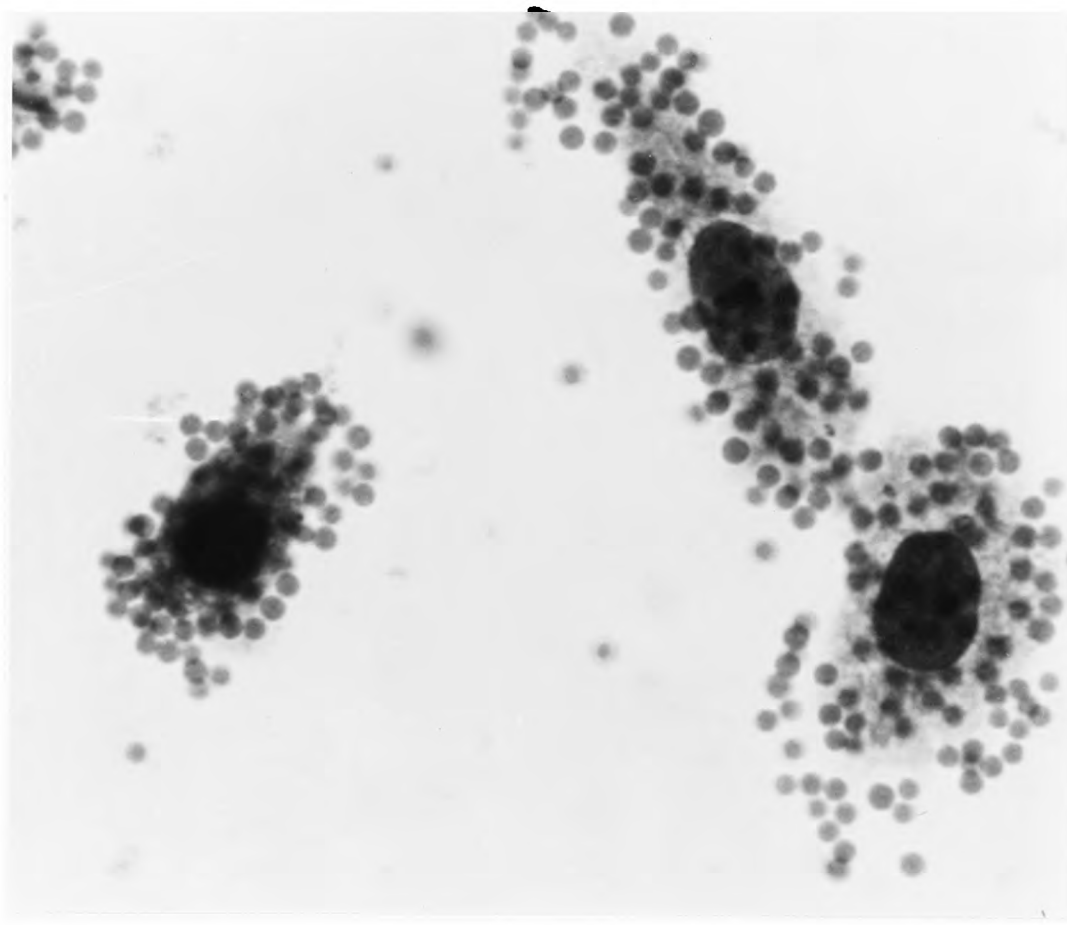

Figure 6. Mixed haemadsorption on HeLa cells with doubly sensitized sheep erythrocytes. HeLa cells were sensitized for 15 minutes with rabbit anti-HeLa serum at a dilution of 1/1,000,000. x 720.

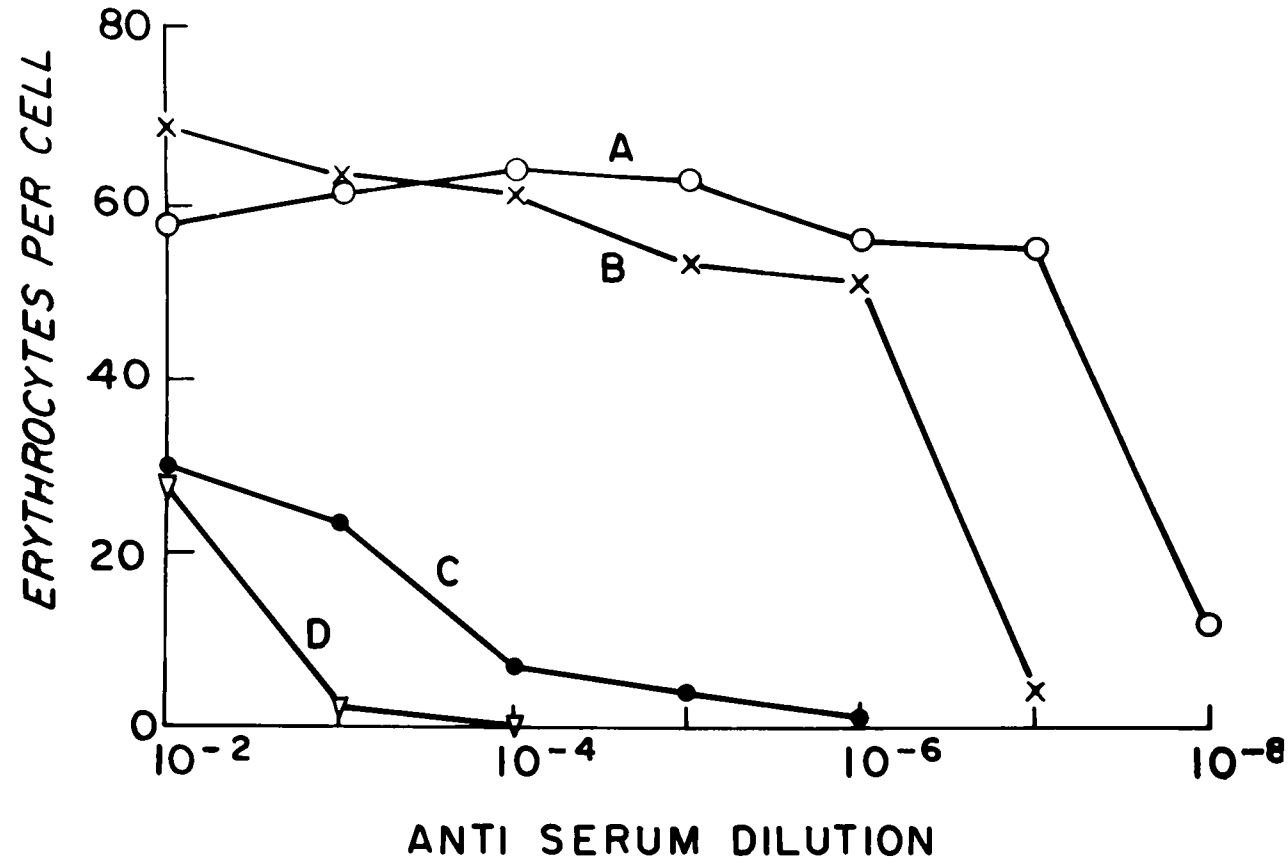

Figure 7. Mixed haemadsorption on HeLa and L cells with rabbit anti-HeLa serum. A - HeLa cells and unabsorbed antiserum. B - HeLa cells and antiserum absorbed with Ehrlich cells. C - L cells and unabsorbed antiserum. D - L cells and Ehrlich-absorbed antiserum.

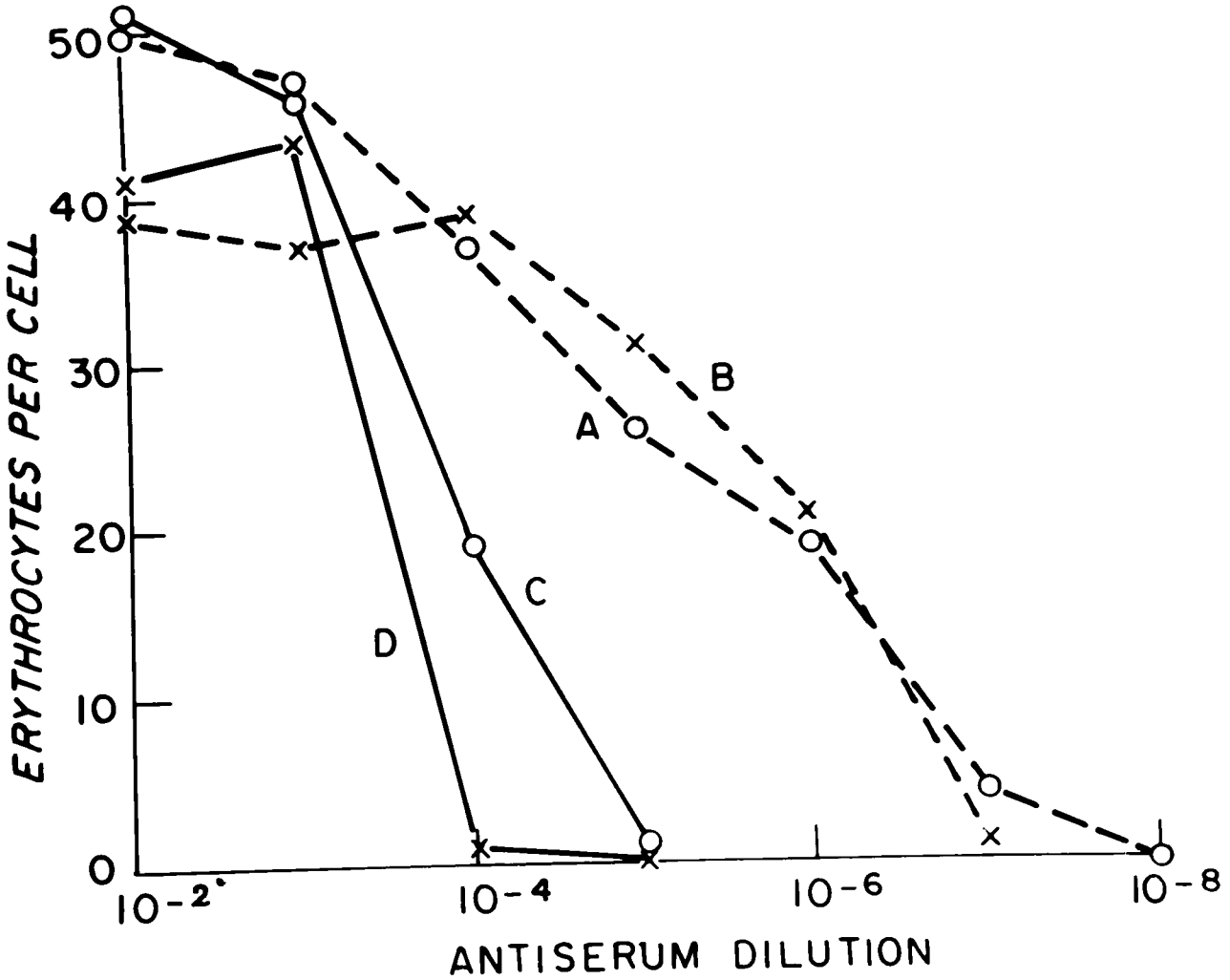

Figure 8. Mixed haemadsorption on HeLa and L cells with rabbit anti-Ehrlich serum. A - L cells and unabsorbed antiserum. B - L cells and antiserum absorbed with HeLa cells. C - HeLa cells and unabsorbed antiserum. D - HeLa cells and HeLa-absorbed antiserum.

Table 3

Effect of Suspending Medium on Adherence of Ehrlich Cells to Coverslips

	Number of adherent cells in:	
	PBS	Culture medium
Ehrlich cells	1.4×10^5	2.1×10^3
Macrophages	2.0×10^4	8.9×10^3

with high concentrations of antiserum, meant that mixed agglutination and immune adherence were not well suited to studies on the surface antigens of heterokaryons. Mixed haemadsorption on the other hand had none of these drawbacks. It gave excellent labelling on tissue culture cells and was extremely sensitive, as shown in Fig. 6. However, with many antisera there was a considerable amount of cross-reaction on other cell lines. The degree of haemadsorption on HeLa and L cells with anti-HeLa and anti-Ehrlich serum, and the effect of absorbing dilute antiserum with heterologous cells, is illustrated in Figs. 7 and 8. The sensitivity of the technique is again shown as well as the difficulty in completely removing cross-reacting antibodies.

Initial haemadsorption experiments were carried out on L cells rather than Ehrlich cells since L cells were more convenient to use. They grow well on coverslips while our strain of Ehrlich ascites cells does not grow in vitro or stick to glass in culture medium. The fact that L cells carry mouse species and transplantation antigens and share many antigens with Ehrlich cells has been discussed in the Introduction. The work of Easty et al. (1960) and of Taylor (1961) suggested that our Ehrlich cells would adhere to glass if they were washed in PBS and allowed to settle from protein-free medium. Table 3 shows the results of such an experiment. Ehrlich cells were washed with PBS and 10^6 cells in 1 ml. of PBS and 10^6 in 1 ml. of culture medium (containing calf serum) were allowed to settle for one hour at room temperature onto coverslips in cups of a haemagglutination tray.

In serum containing medium very few Ehrlich cells adhered to the coverslip and these were easily washed off, while in PBS many cells

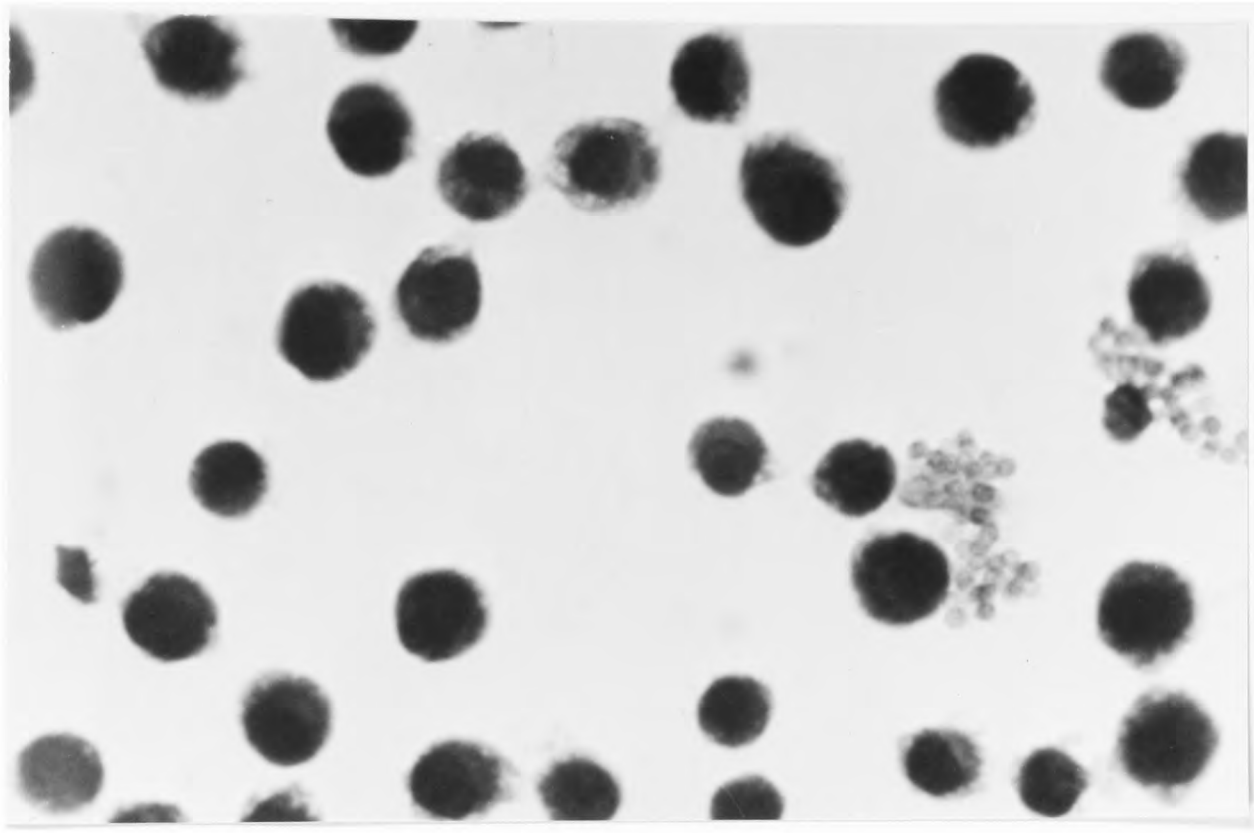

Figure 9. Mixed haemadsorption on Ehrlich cell monolayer with Ehrlich-absorbed anti-HeLa serum at a concentration of 1/1,000. All Ehrlich cells are unlabelled. The only haemadsorption is on macrophages which adsorb doubly sensitized erythrocytes. x 300.

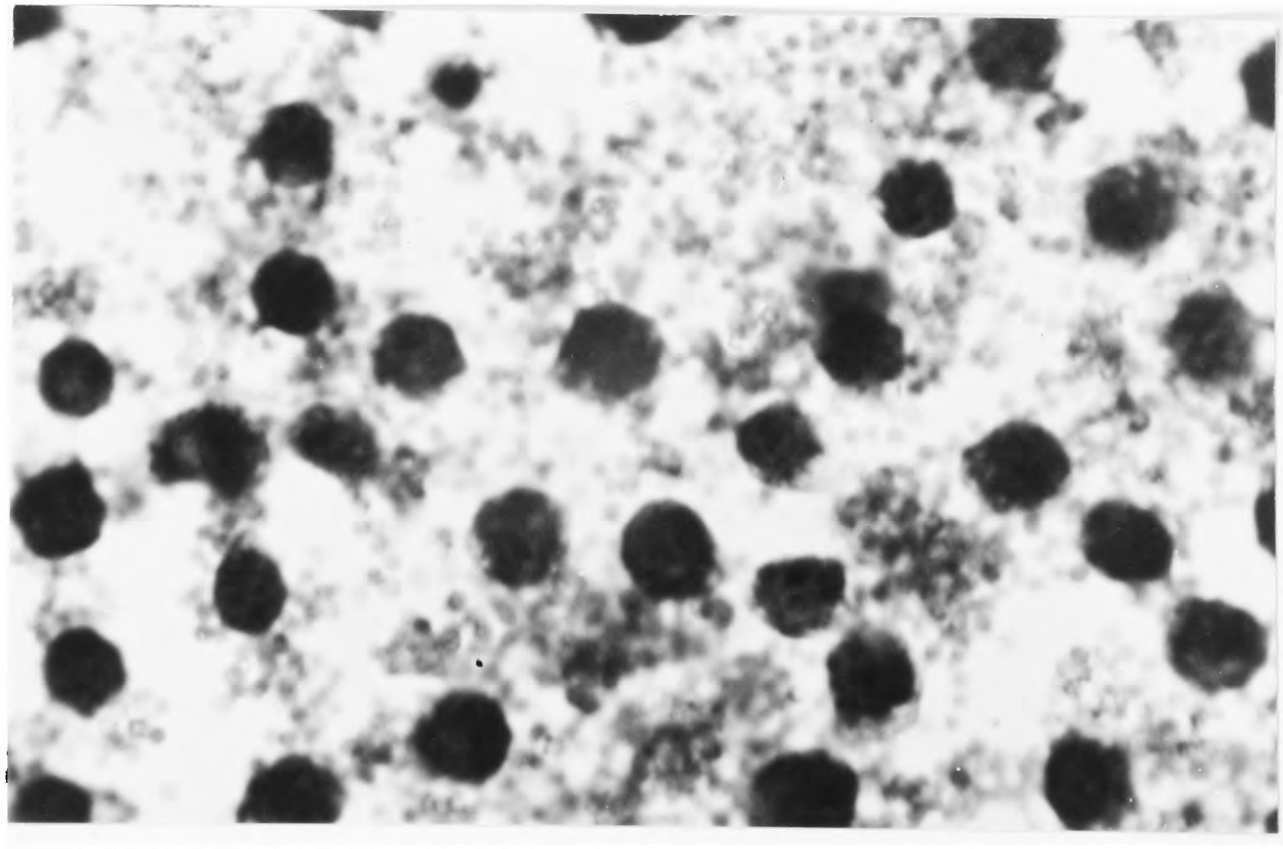

Figure 10. Mixed haemadsorption on Ehrlich cell monolayer with HeLa-absorbed anti-Ehrlich serum at a concentration of 1/10,000. All Ehrlich cells are strongly labelled. x 300.

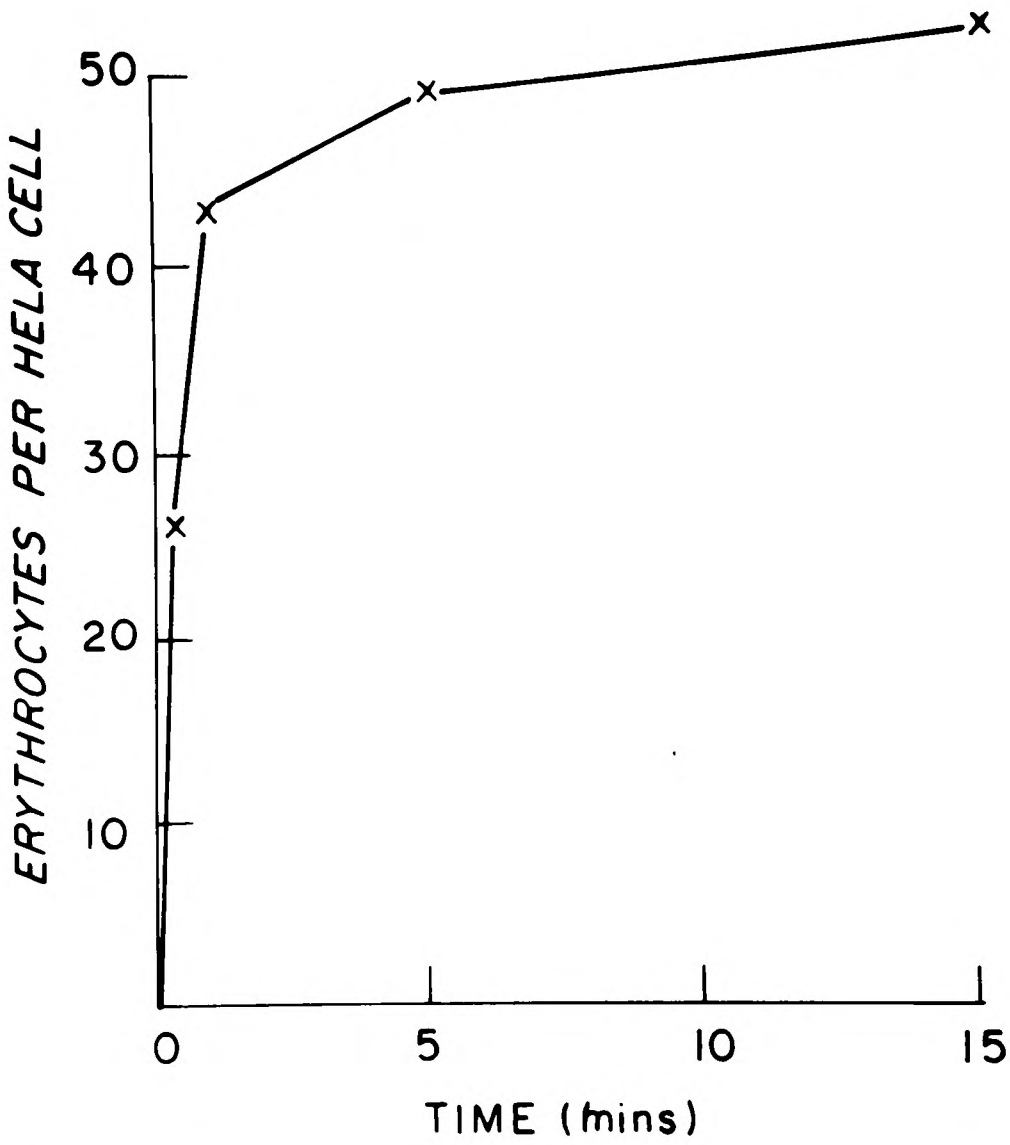

Figure 11. Mixed haemadsorption on HeLa cells with increasing time of antibody sensitization. Rabbit anti-HeLa serum was used at a concentration of 1/1,000. The first two points on the graph represent 15 and 60 seconds.

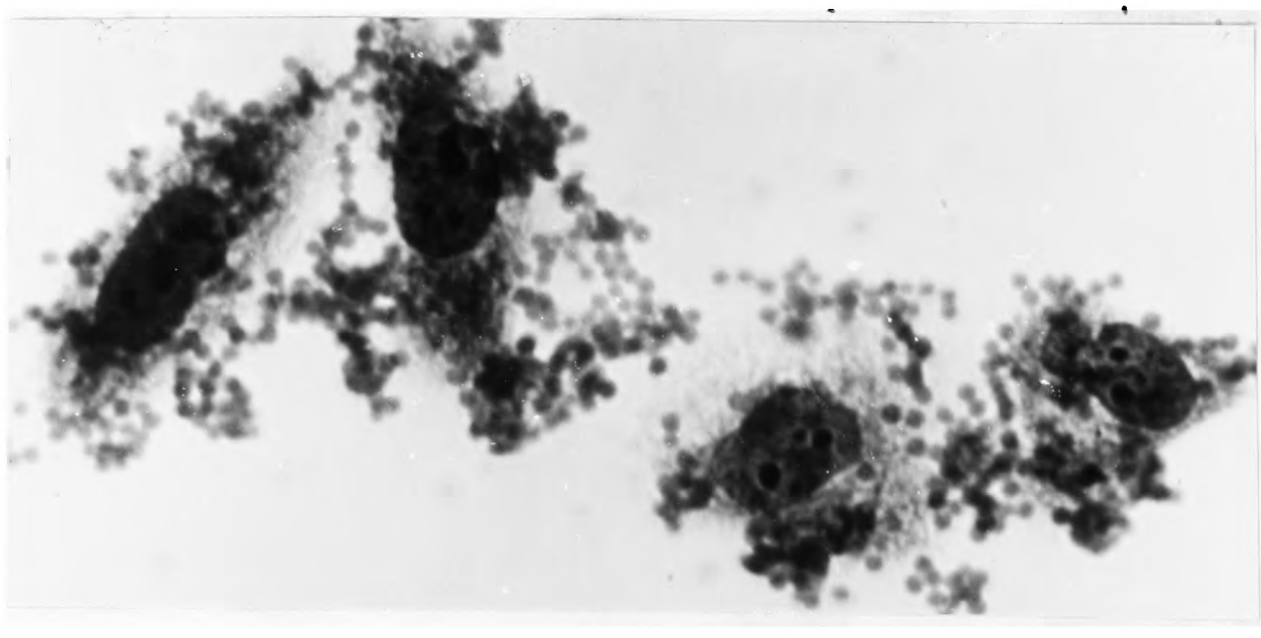

Figure 12. Mixed haemadsorption on HeLa cells after 15 seconds of sensitization with rabbit anti-HeLa serum diluted 1/1,000. x 720.

stuck and remained adherent for several hours if the protein content of the suspending medium was low. Serum also decreased the rate of macrophage adherence though to a much smaller extent. The specificity of antiserum established in mixed haemadsorption experiments with L cell monolayers was confirmed with Ehrlich ascites cell monolayers produced with this method. Figs. 9 and 10 show haemadsorption on Ehrlich cells with absorbed anti-HeLa and anti-Ehrlich serum.

Workers using the mixed haemadsorption reaction incubated cell monolayers in antiserum dilutions for a long time. Sensitisation of tissue culture cells was carried out for 1 hour by Fagraeus et al. (1965), for 2 hours by Milgrom et al.(1964), and for 3 hours by Milgrom et al. (1965). In view of the great sensitivity of the reaction, it was of interest to see if such prolonged sensitisation was necessary. Fig. 11 shows the variation in haemadsorption with time of exposure of HeLa cells to anti-HeLa serum. Considerable haemadsorption was obtained on HeLa cells after only 15 seconds of sensitisation at room temperature as illustrated in Fig. 12.

Excellent haemadsorption could be obtained with concentrations of antisera which used alone had no effect on cell viability. It was of interest to see if the adherence of red cells affected cell multiplication since mixed haemadsorption might be of assistance in identifying and isolating clones of cells in a mixed population. 5×10^5 HeLa cells in culture medium were placed in a Petri dish containing coverslips. Twenty-four hours later coverslips with adherent HeLa cells were exposed to anti-HeLa serum at a concentration of 10^{-3} for 15 minutes,

Table 4

Growth of HeLa Cells with Adherent Erythrocytes

Days after mixed haemadsorption	Number of cells per coverslip	
	Control (No anti-serum or erythro-cytes)	Cells labelled with erythrocytes
1	4.3×10^4	4.1×10^4
2	9.6×10^4	8.1×10^4
3	1.3×10^5	1.4×10^5

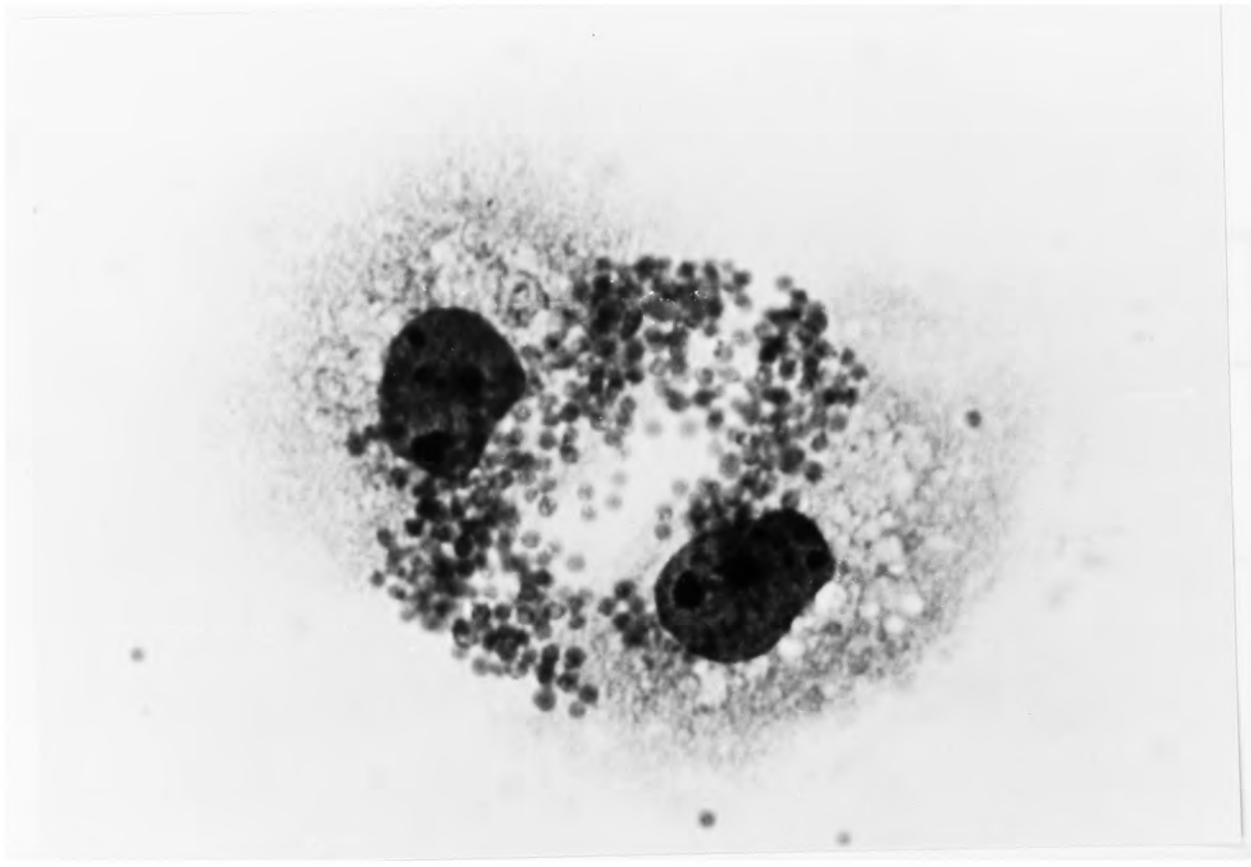

Figure 13. Pair of HeLa cells 24 hours after being labelled on a monolayer with doubly sensitized erythrocytes. Anti-HeLa serum had been used at a concentration of 1/1,000. x 600.

rinsed in PBS, and placed in the cup of a haemagglutination tray with 0.5% doubly sensitised erythrocytes. The coverslips were then rinsed in 3 changes of PBS and incubated in culture medium. Daily counts of HeLa cells were made and the results are shown in Table 4. Culture medium was changed daily.

It can be seen that cells labelled with erythrocytes multiplied as well as unlabelled controls. One day after labelling virtually all HeLa cells were still haemadsorption positive and after 3 days there were still a number of patches of erythrocytes over confluent areas of HeLa cells. Most HeLa cells remained normal in morphology although a few did appear vacuolated. It was noted that erythrocytes often assumed a position between adjacent HeLa cells 1 or 2 days after labelling as shown in Fig. 13.

Mixed haemadsorption was also carried out with cells in suspension. Trypsinised HeLa cells were exposed to anti-HeLa serum at a concentration of 10^{-3} in Hanks' solution. After 15 minutes of incubation the cells were washed 3 times in PBS. Doubly sensitised erythrocytes to a final concentration of 0.5% were added and the cells centrifuged out at 500 g. The cells were resuspended in Hanks' solution and examined in a counting chamber. Haemadsorption on HeLa cells was obtained but was not as strong or as reliable as on monolayer cells. Moreover, haemadsorption all over suspended cells made examination difficult. This would be a particular problem with heterokaryons since nuclear type must be noted.

(iv) Viral haemadsorption

The problem of viral haemadsorption on heterokaryons and the method

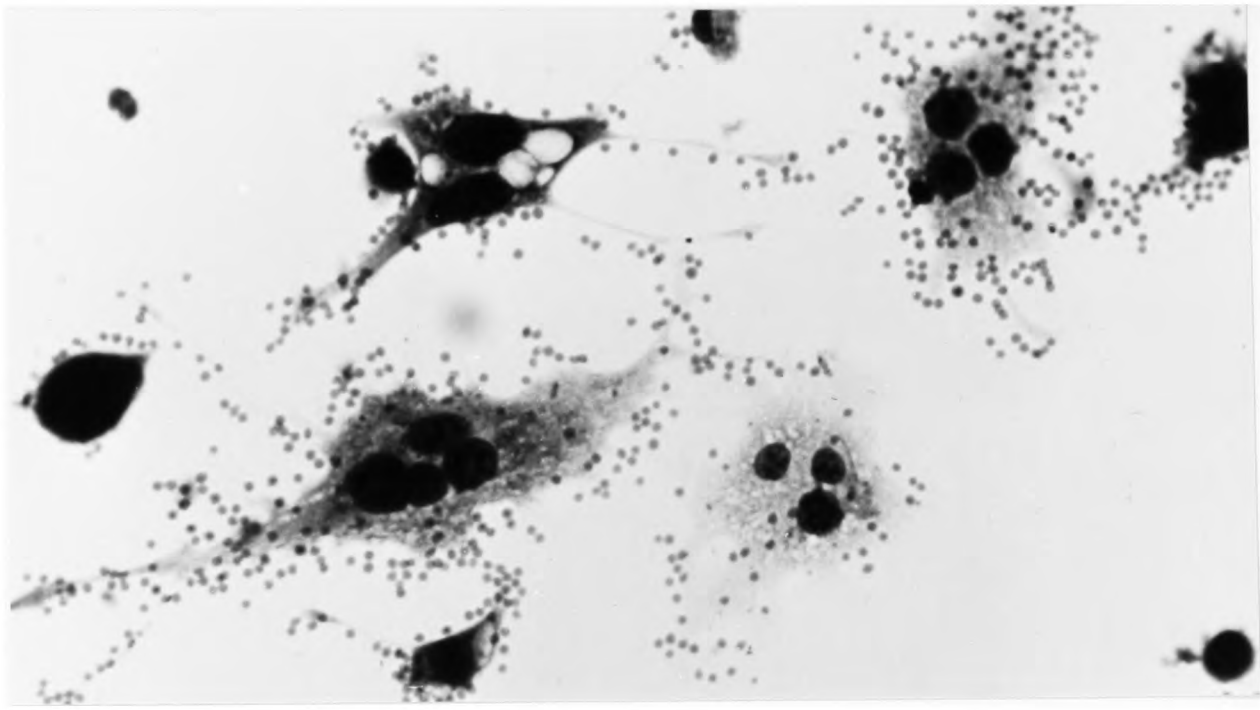

Figure 14. Viral haemadsorption of sheep erythrocytes to HeLa-Ehrlich heterokaryons 24 hours after fusion with 4,000 HAU of UV-inactivated Sendai virus. x 250.

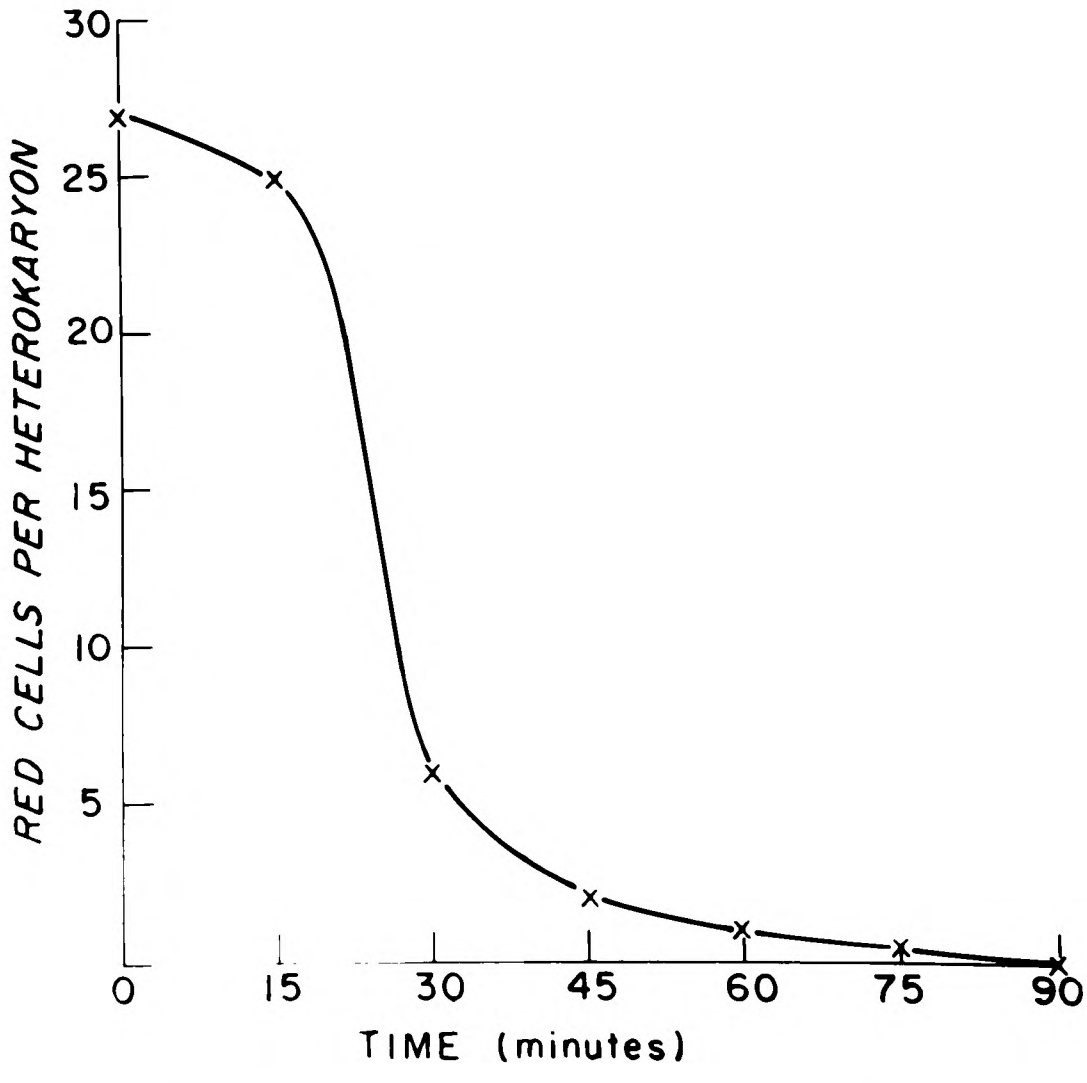

Figure 15. Effect of post-incubation of heterokaryon cultures at 37°C on the degree of viral haemadsorption.

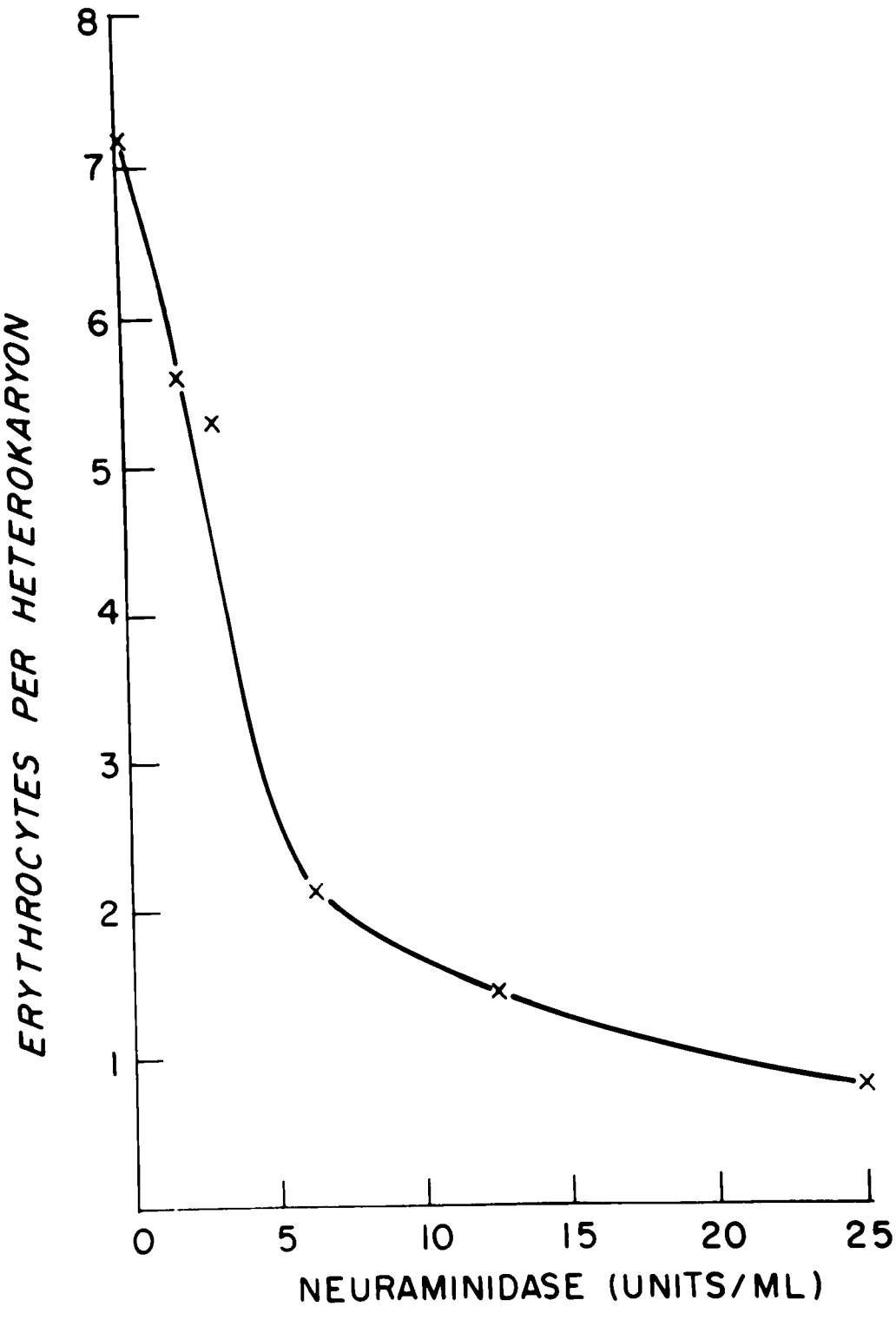

Figure 16. Effect of neuraminidase treatment of sheep erythrocytes on the degree of viral haemadsorption on HeLa-Ehrlich heterokaryons one day after fusion.

of overcoming this problem by post-incubation of coverslips in culture medium after mixed haemadsorption has been discussed briefly under Materials and Methods. Such viral haemadsorption is illustrated in Fig. 14, and the effect of post-incubation in Fig. 15. Before this simple procedure was discovered, an attempt was made to find a type of erythrocyte which would not show viral haemadsorption. Human, sheep, mouse, horse, hen, duck, pigeon, and frog erythrocytes all adsorbed to virus at the cell surface but cat erythrocytes did not show this response and were only very weakly agglutinated by virus when in suspension. Neuraminidase treatment of erythrocytes can also be used to decrease viral haemadsorption as shown in Fig. 16. 1% sheep erythrocytes in Hanks' solution were treated with neuraminidase for 1 hour at 37°C.

2. Mouse Peritoneal Macrophage Heterokaryons

(i) Ehrlich-macrophage heterokaryons

A suspension of Ehrlich ascites cells freshly withdrawn from the peritoneal cavity of the mouse may contain up to 20% macrophages. These fuse readily with tumour cells when treated with Sendai virus and the resultant heterokaryons stick to glass. If the individual nuclei in such heterokaryons followed the usual sequence of synchronous mitosis and reconstitution as a single nucleus, the result would be a mononuclear cell sticking to glass and giving positive mixed haemadsorption with anti-Ehrlich serum. It was hoped to identify HeLa-Ehrlich heterokaryons by the fact that they possessed Ehrlich surface antigens

and stuck to glass since single Ehrlich cells would not stick unless fused with a HeLa cell. A mononuclear cell formed by fusion of an Ehrlich cell and a mouse macrophage would thus be indistinguishable from a HeLa-Ehrlich heterosynkaryon. A further difficulty could arise from the fact that mouse peritoneal macrophages adsorb erythrocytes sensitised with rabbit anti-erythrocyte serum, as discussed in the Introduction, and also adsorb the doubly sensitised erythrocytes and bacteria used in the present experiments. This property might persist in an Ehrlich-macrophage heterokaryon which would then appear positive after treatment with anti-HeLa serum. Problems of interpretation could also arise from fusion of HeLa cells with mouse peritoneal macrophages.

Ehrlich-mouse macrophage heterokaryons formed by fusing 10^7 Ehrlich cells and 10^7 macrophages had the following characteristics:

1. The number of heterokaryons on the coverslips decreased rapidly, falling in a typical experiment from 1.3×10^4 per coverslip 24 hours after fusion to 14 per coverslip 3 days after fusion. The numbers of unfused macrophages remained fairly constant. These heterokaryons, therefore, survive for only a short period.

2. The nuclei remained discrete throughout the life of the heterokaryon, and no synkaryons were seen. The macrophage nucleus enlarged, until in some cases it was the same size as the Ehrlich nucleus, and developed 1 or 2 well-marked nucleoli. These heterokaryons were at all times distinguishable from HeLa-Ehrlich heterokaryons. The Ehrlich-macrophage heterokaryons degenerated in a characteristic way. They remained flattened and showed pyknosis of their nuclei; the cytoplasm

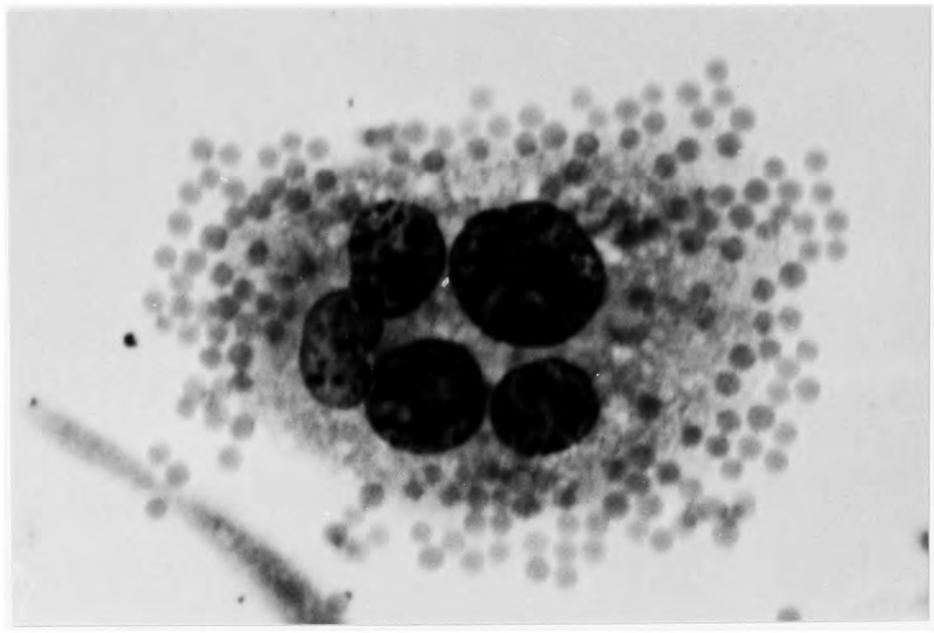

Figure 17. Ehrlich-mouse macrophage heterokaryon showing adsorption of sensitized erythrocytes. The heterokaryon contains one macrophage nucleus on the left side and four Ehrlich nuclei. x 720.

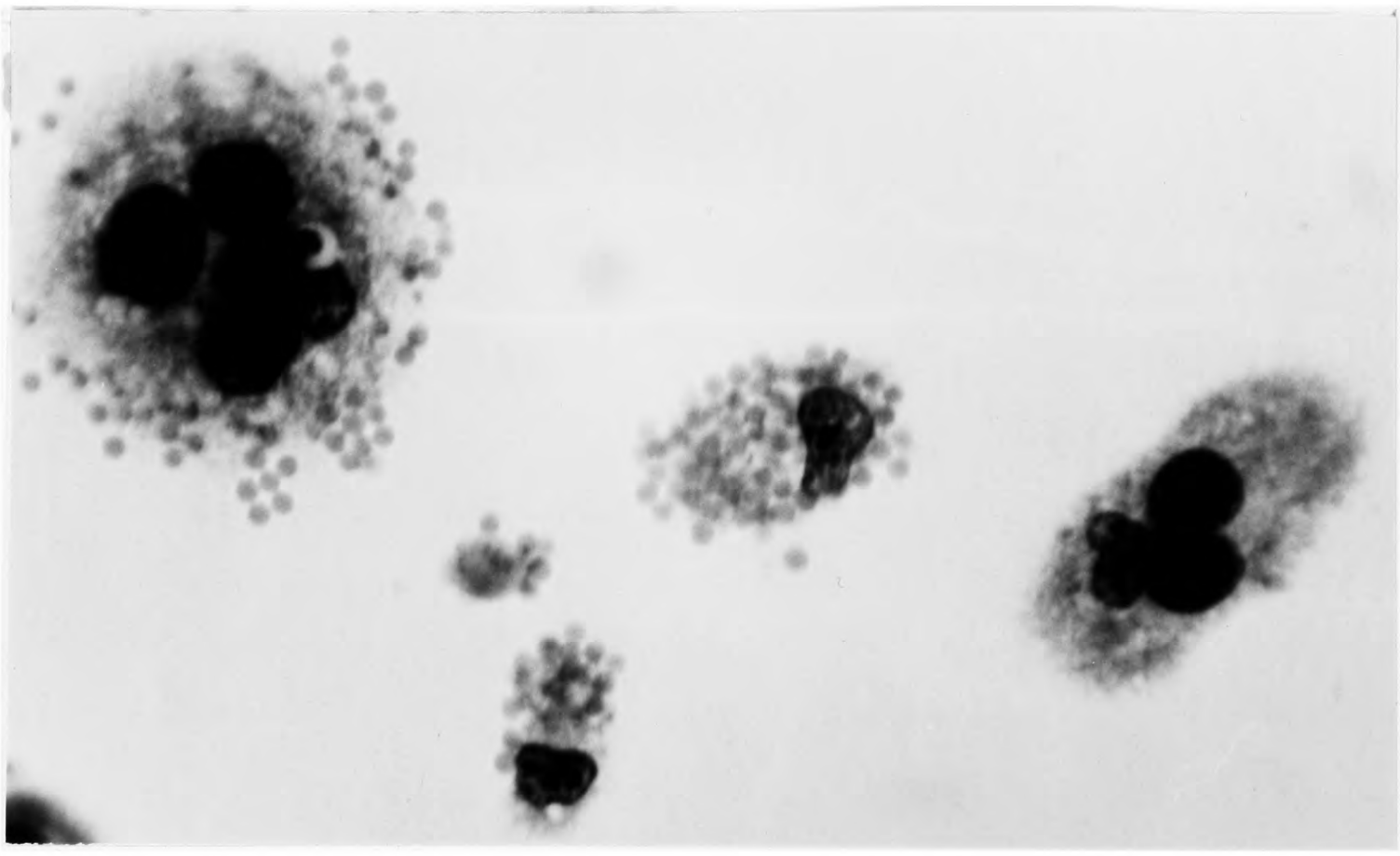

Figure 18. Ehrlich-mouse macrophage heterokaryons (left and right) with two single macrophages (centre). One heterokaryon has lost the macrophage property of adsorbing sensitized sheep erythrocytes. x 600.

became eosinophilic and granular before the cell came off the glass.

3. Macrophage-type haemadsorption of singly sensitised erythrocytes was shown by about 80% of these heterokaryons 24 hours after fusion. Fig. 17 shows this type of haemadsorption on an Ehrlich-macrophage heterokaryon. It is of interest that the heterokaryon shown contains only one macrophage nucleus with 4 Ehrlich nuclei and yet still has the macrophage properties of sticking to glass and adsorbing singly sensitised erythrocytes. Fig. 18 shows some Ehrlich-macrophage heterokaryons and single macrophages. Two heterokaryons are adsorbing singly sensitised erythrocytes while a third heterokaryon has lost this property.

These heterokaryons did not show any phagocytosis of carbon particles in culture medium although single macrophages also did not phagocytose well while adherent to a coverslip.

(ii) HeLa-macrophage heterokaryons

Unlike rabbit peritoneal macrophages, mouse macrophages did not fuse readily with HeLa cells. After treatment of 10^7 HeLa cells and 10^7 mouse peritoneal macrophages with 8,000 HAU of virus less than 1% of multinucleate cells contained a macrophage nucleus, and the few heterokaryons present did not show macrophage-type haemadsorption.

HeLa-macrophage and Ehrlich-macrophage heterokaryons thus did not constitute a serious source of error in experiments on HeLa-Ehrlich heterokaryons. However, in some experiments the macrophage content of Ehrlich cell suspensions was nonetheless reduced by allowing the suspension to settle in a plastic Petri dish for one hour at $37^{\circ}C$.

Table 5

Mixed Haemadsorption on HeLa-Ehrlich Heterokaryons with Dilute Anti-Ehrlich Serum

Distribution of nuclei	Range of nuclei (Ehrlich to HeLa)	Haemadsorption +ve heterokaryons (%)
Ehrlich > HeLa	2/1 to 10/3	97
Ehrlich = HeLa	1/1 to 6/6	87
Ehrlich < HeLa	1/2 to 1/6	66

The macrophages adhered to the floor of the dish, whereas the Ehrlich cells were readily resuspended.

3. HeLa-Ehrlich Heterokaryons

(i) Mixed haemadsorption

Initial haemadsorption experiments were carried out with unabsorbed anti-Ehrlich serum at a concentration of 10^{-6}. This dilution was used to obtain complete specificity before absorbed serum, which was specific at higher concentrations, was available. 10^{6} HeLa cells and 10^{7} Ehrlich cells were fused with 8,000 HAU of virus and haemadsorption carried out with unabsorbed serum. The results are summarised in Table 5. A heterokaryon with more than 10 adsorbed erythrocytes has been counted as positive. This experiment shows that the amount of antigen on the surface of heterokaryons is, as one might expect, proportional to the ratio of Ehrlich to HeLa nuclei. Most heterokaryons unlabelled for Ehrlich antigen had an excess of HeLa nuclei.

With specific absorbed anti-Ehrlich serum at a concentration of 5×10^{-3} all multinucleated cells which contained recognisable Ehrlich nuclei showed haemadsorption. All heterokaryons were also labelled with rabbit anti-HeLa serum at a concentration of 10^{-3}. In heterokaryons, nuclear fusion occurs progressively, often with the formation of nuclei of bizarre shape (Harris and Watkins, 1965). All cells with bizarre nuclei showed haemadsorption with rabbit anti-HeLa serum, in one experiment 3 weeks after fusion, but not all were positive with anti-Ehrlich serum. There was, of course, no means of telling whether cells with

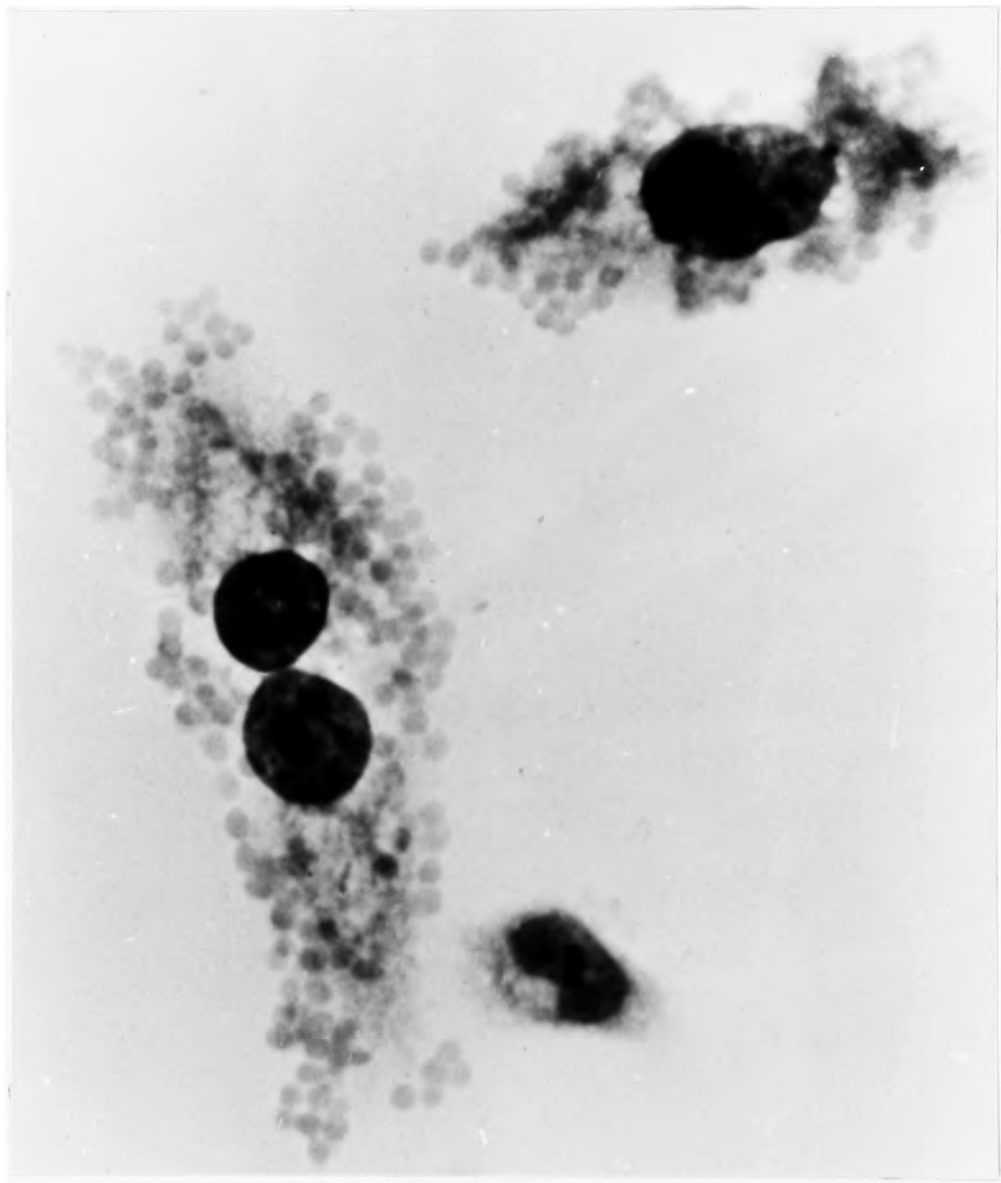

Figure 19. Mixed haemadsorption with rabbit anti-Ehrlich serum 24 hours after the fusion of HeLa and Ehrlich cells. The binucleate cell contains one HeLa and one Ehrlich nucleus and is labelled over its whole surface for Ehrlich antigens. The mononucleate cell must be a heterosynkaryon since it is labelled for Ehrlich antigens but has the HeLa surface property of sticking to glass. A single HeLa cell is also present. x 1,000.

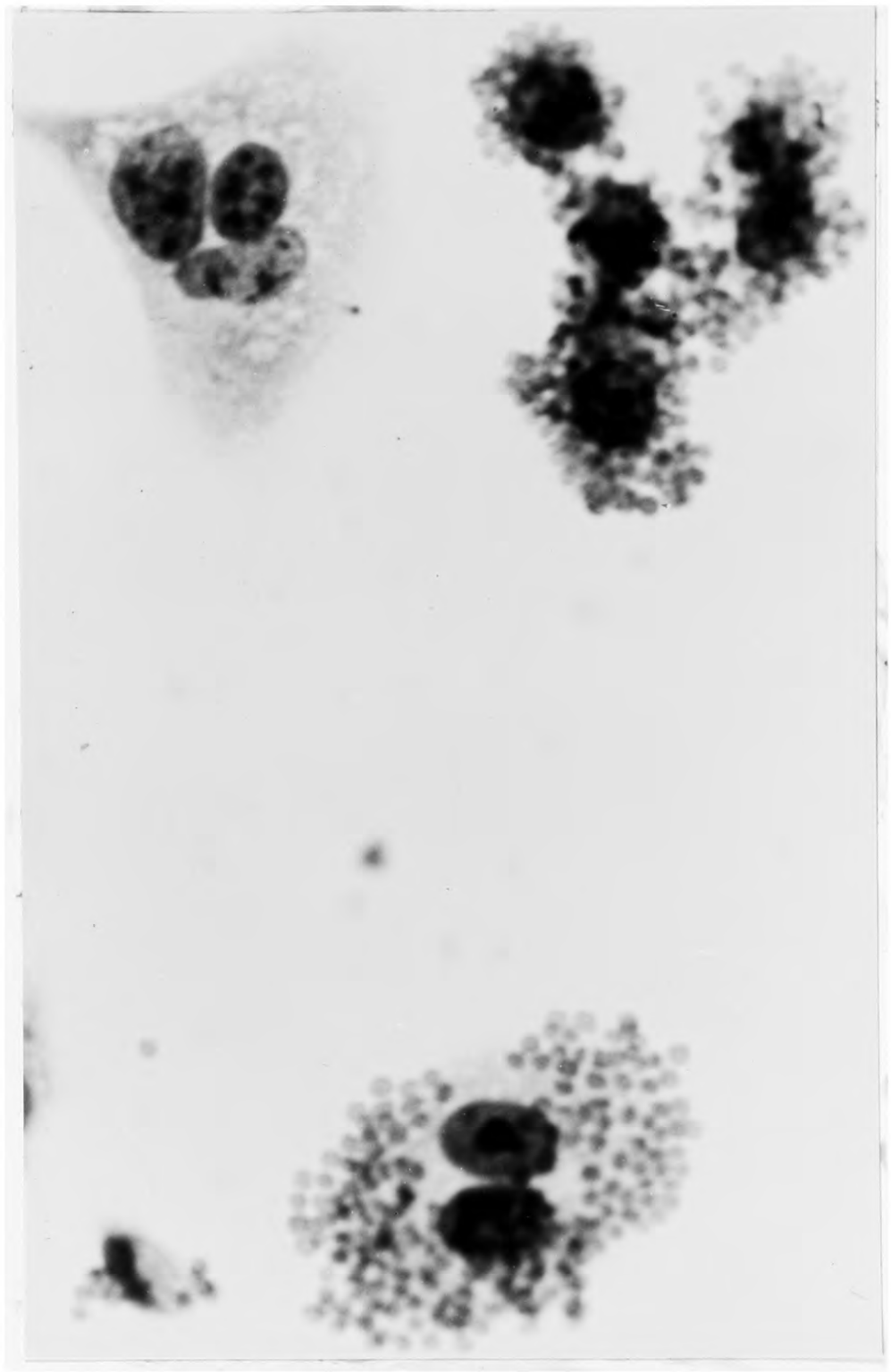

Figure 20. HeLa-Ehrlich heterokaryon (bottom right) labelled for HeLa antigens 2 days after cell fusion. A group of labelled HeLa cells is also present (top right). In order to show the specificity of the antiserum macrophages were not removed from this preparation. An Ehrlich-macrophage heterokaryon is present (top left) and is unlabelled with anti-HeLa serum. It has lost the macrophage property of adsorbing sensitized erythrocytes which is shown by the single macrophage (bottom left). x 600.

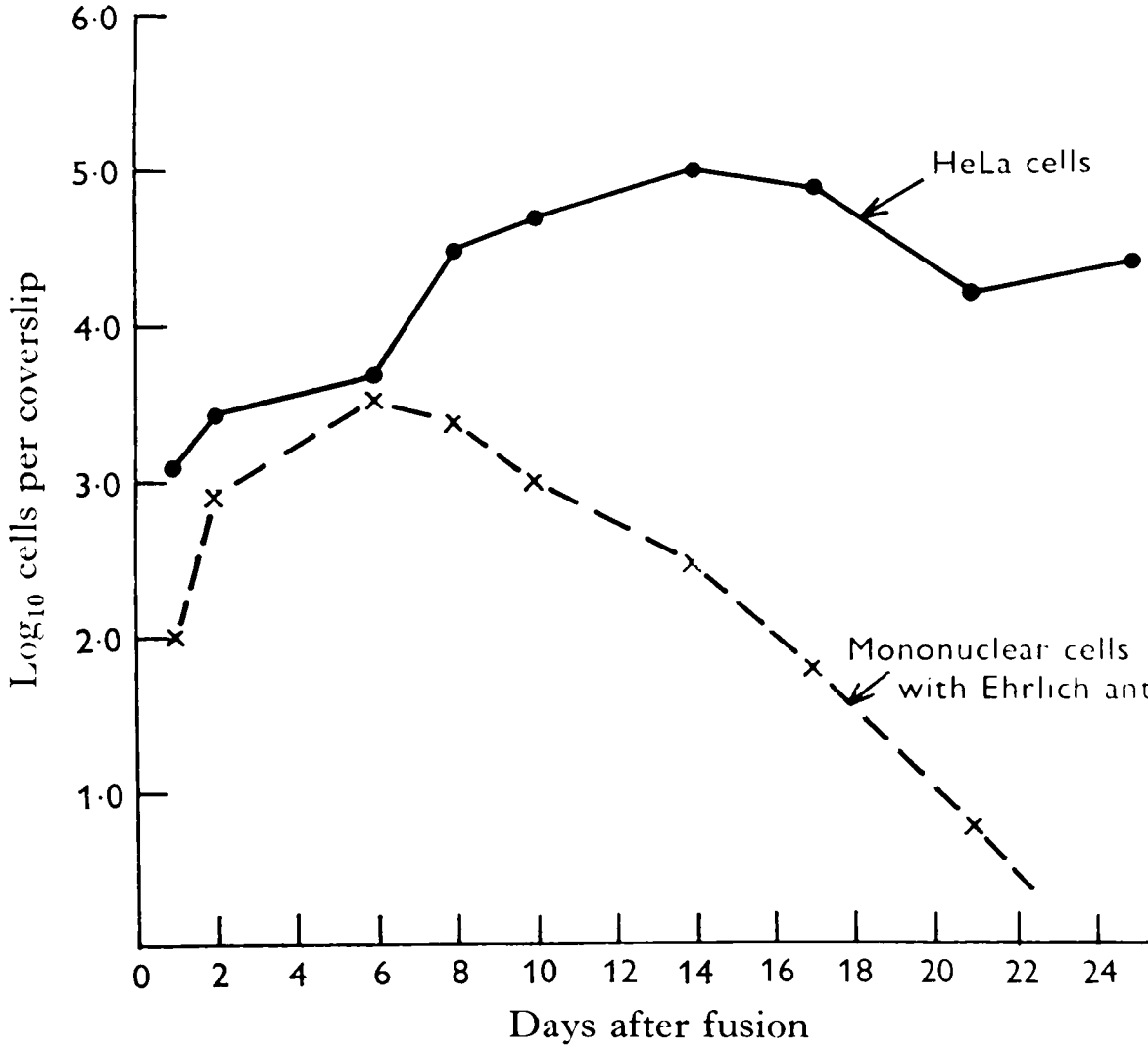

Figure 21. The number of mononuclear cells labelled for Ehrlich antigens and single HeLa cells for 25 days after cell fusion. The decrease in HeLa cells 17 days after fusion was due to overcrowding of the monolayer.

bizarre nuclei that were negative with anti-Ehrlich serum contained Ehrlich chromosomes or were HeLa-HeLa homosynkaryons. It is clear, however, that many of the heterokaryons carried the surface antigens of each species for prolonged periods. The antigens of each species appeared to be fairly evenly mixed since haemadsorption with each antiserum was distributed over the whole cell surface (Figs. 19 and 20).

All mononuclear cells showed haemadsorption with antiserum against HeLa cells throughout the duration of each experiment, in one case 20 days after fusion. These cells included typical HeLa cells which gradually overgrew the culture as well as cells with large atypical nuclei which might have been heterosynkaryons or HeLa homosynkaryons. That many of them were indeed heterosynkaryons was shown by the fact that in all experiments mononuclear cells showing haemadsorption with anti-Ehrlich serum were present from one day after fusion. As described by Harris and Watkins (1965) hybrid cells showed no tendency to multiply under the usual cultural conditions when considered en masse. This fact is illustrated by Fig. 21, a graph derived from an experiment in which counts were made at intervals of the numbers of mononuclear cells labelled with anti-Ehrlich serum and the number of HeLa cells per coverslip. The initial rise in numbers of mononuclear cells with Ehrlich antigens is largely attributable to nuclear fusion in heterokaryons and does not necessarily imply multiplication. There was evidence, however, that some of these cells were capable of at least some multiplication since many pairs or small groups of fibroblastic

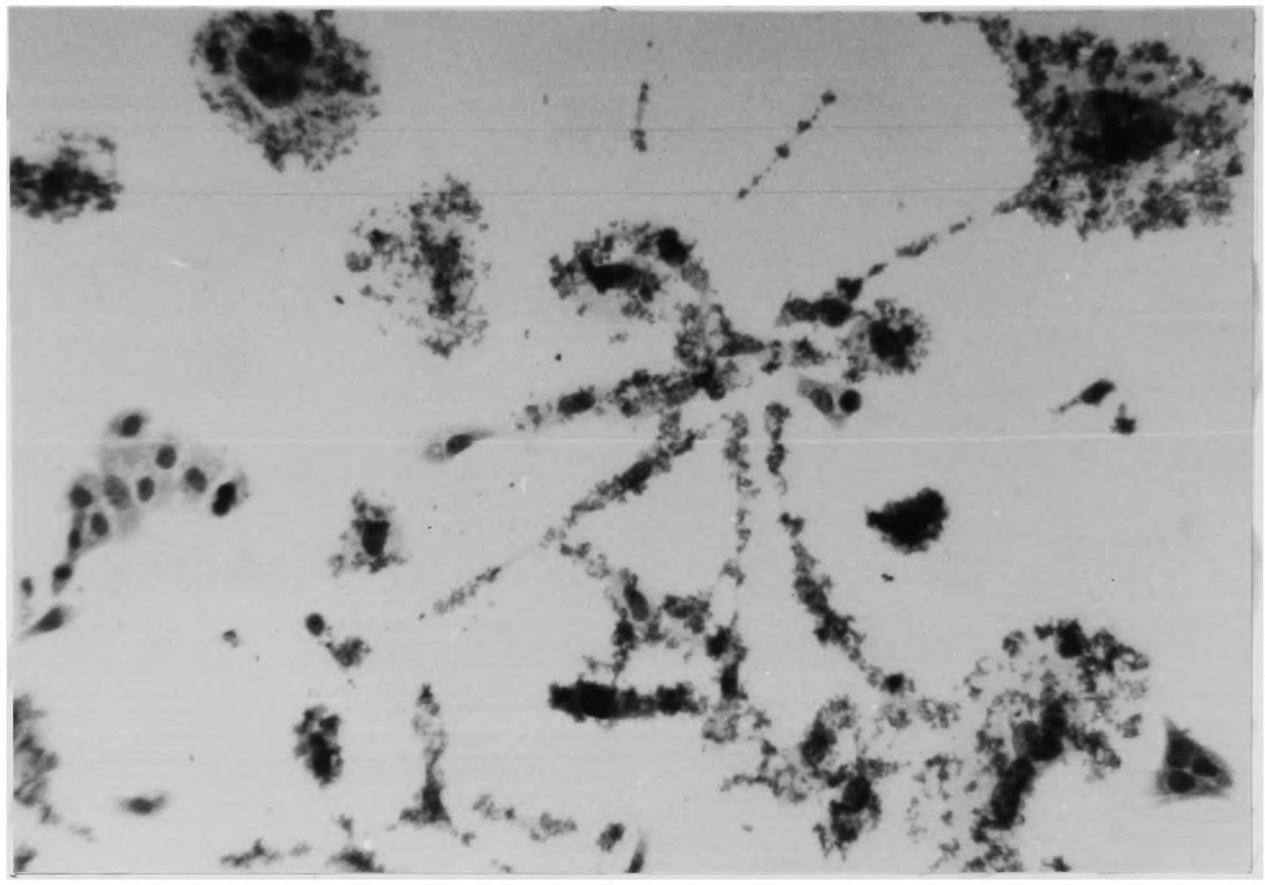

Figure 22. HeLa-Ehrlich heterokaryons labelled for Ehrlich antigens by mixed haemadsorption 6 days after cell fusion. A number of fibroblastic heterosynkaryons are seen as well as larger heterokaryons and a group of unlabelled HeLa cells. x 120.

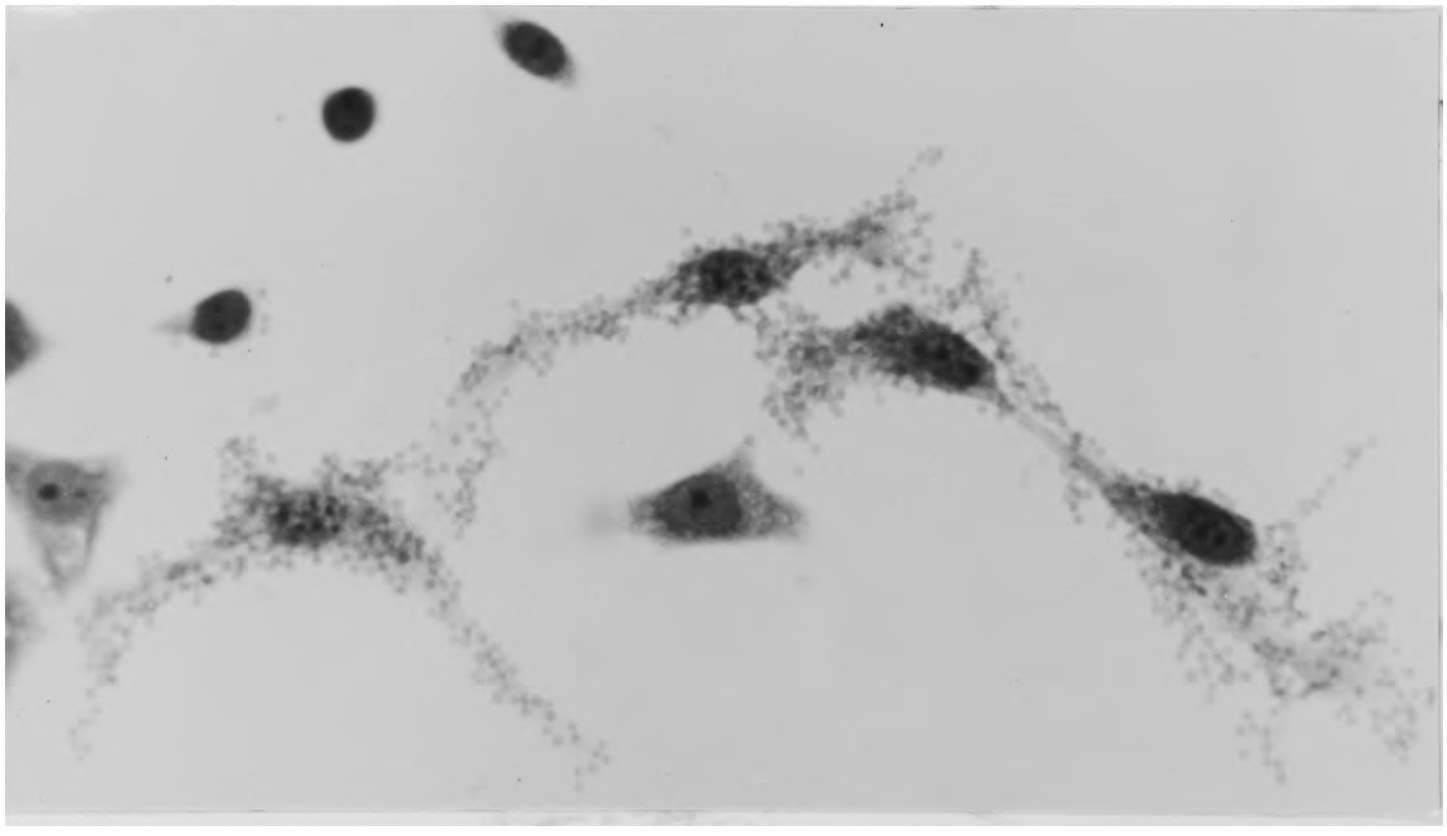

Figure 23. Group of fibroblastic HeLa-Ehrlich heterosynkaryons labelled for Ehrlich antigens 4 days after cell fusion. x 400.

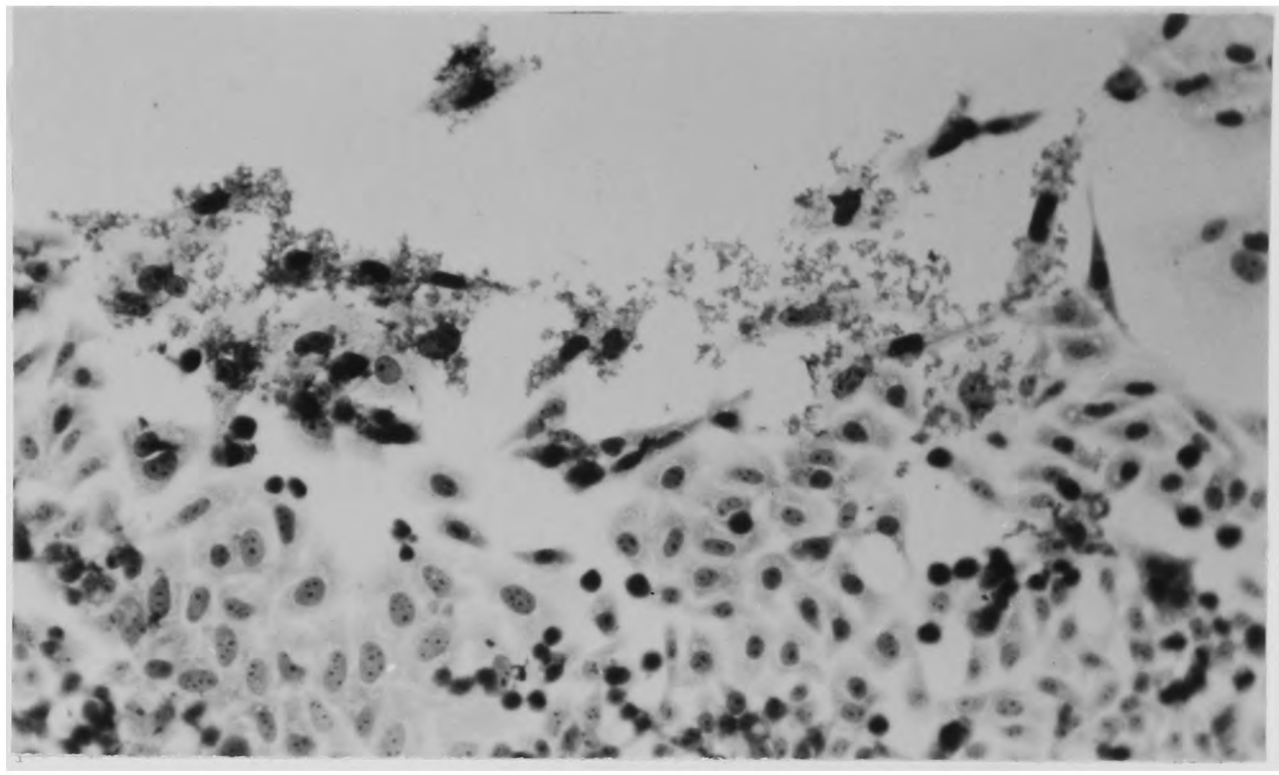

Figure 24. Group of HeLa-Ehrlich heterokaryons labelled for Ehrlich antigens 17 days after fusion. A number of unlabelled HeLa cells are also present. x 120.

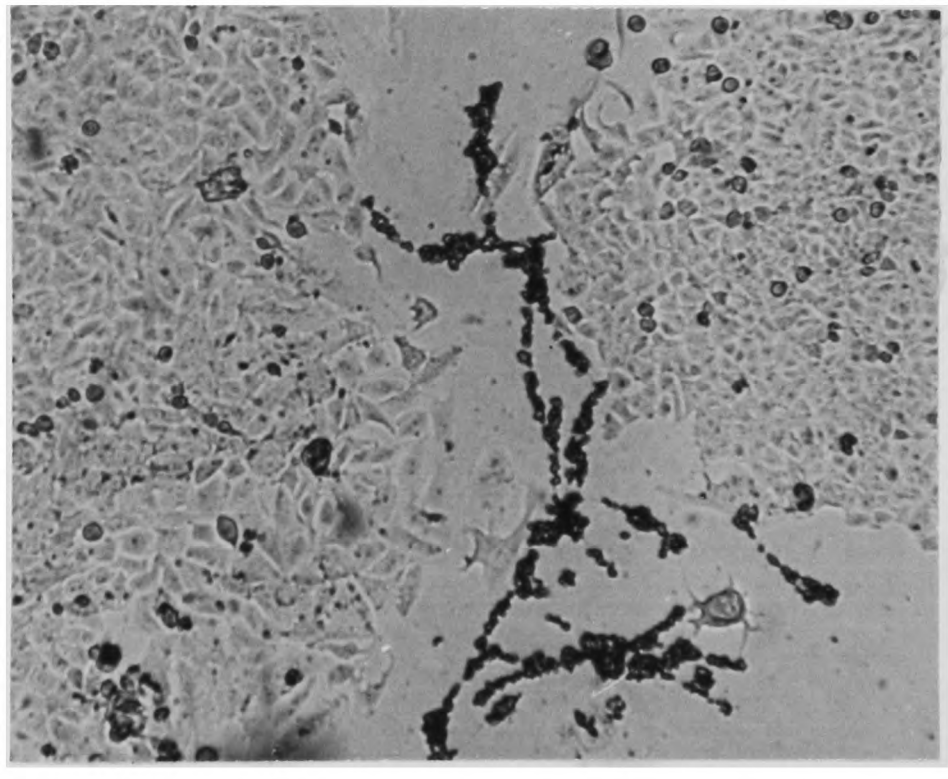

Figure 25. Unfixed preparation showing a clone of cells labelled for Ehrlich antigens. These appear dark due to the adsorbed erythrocytes. x 50.

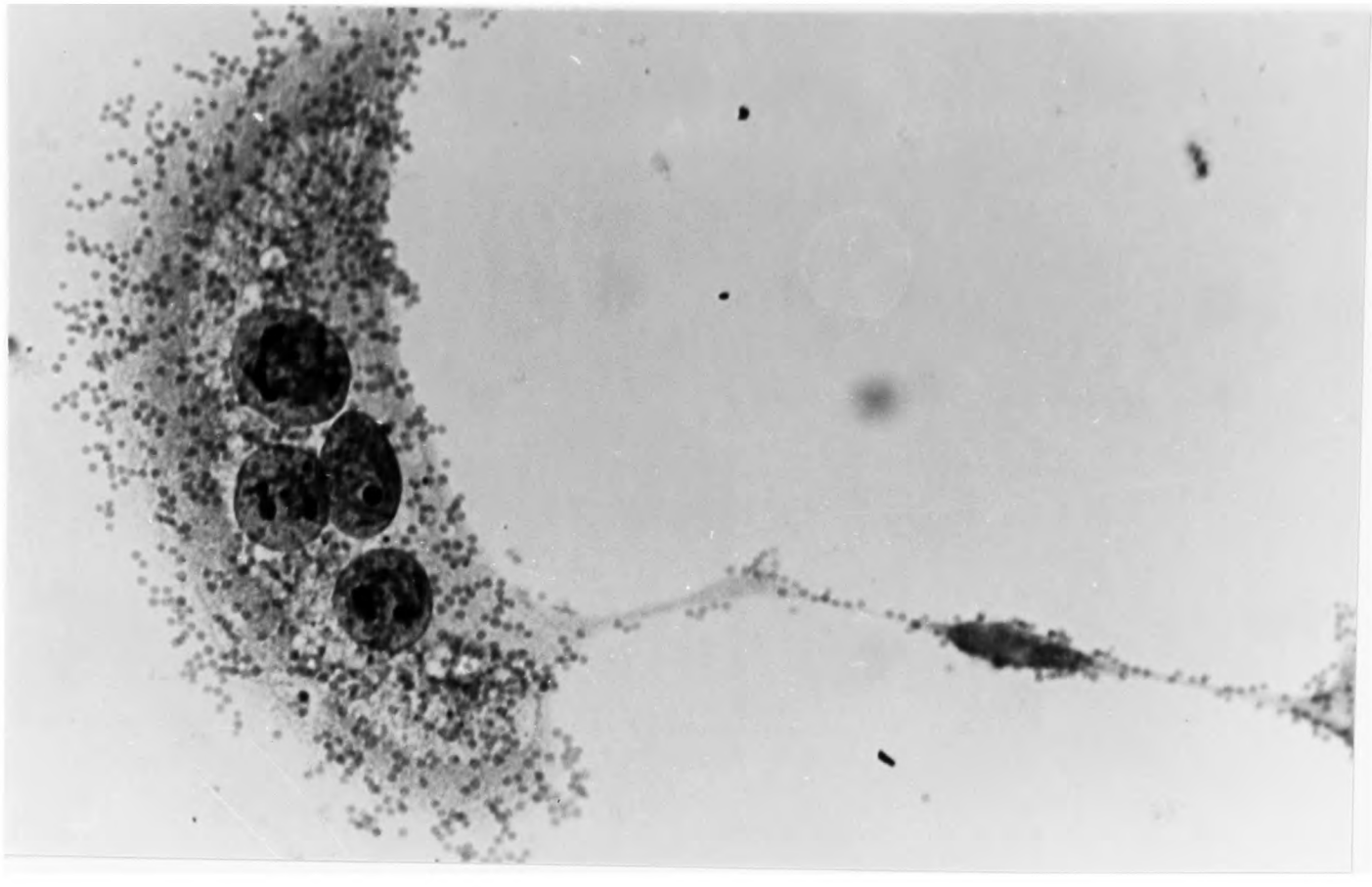

Figure 26. HeLa-Ehrlich heterokaryon labelled for Ehrlich antigens 4 days after fusion. There is an unusual cytoplasmic projection. x 300.

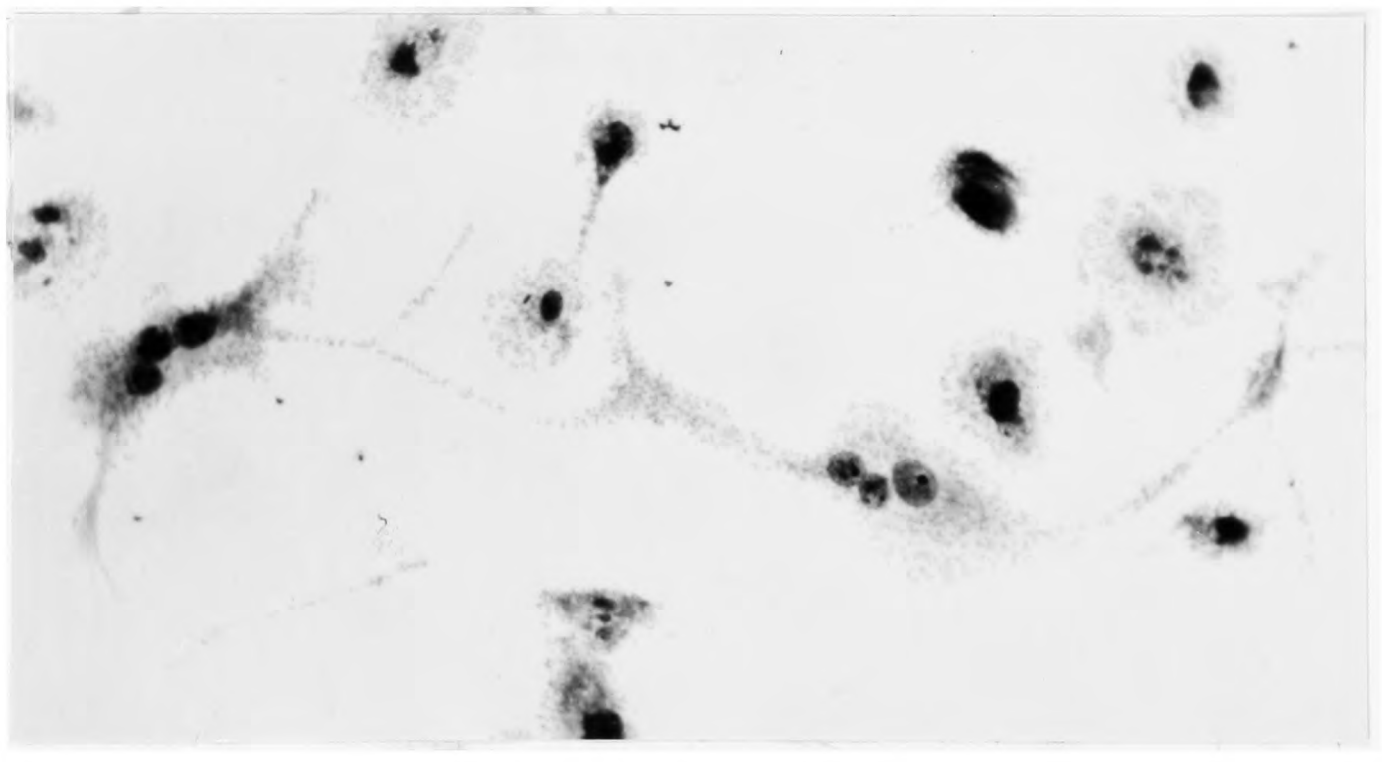

Figure 27. HeLa-Ehrlich heterokaryon labelled for HeLa antigens 4 days after fusion. The cell has an unusual shape and 2 groupings of nuclei. x 130.

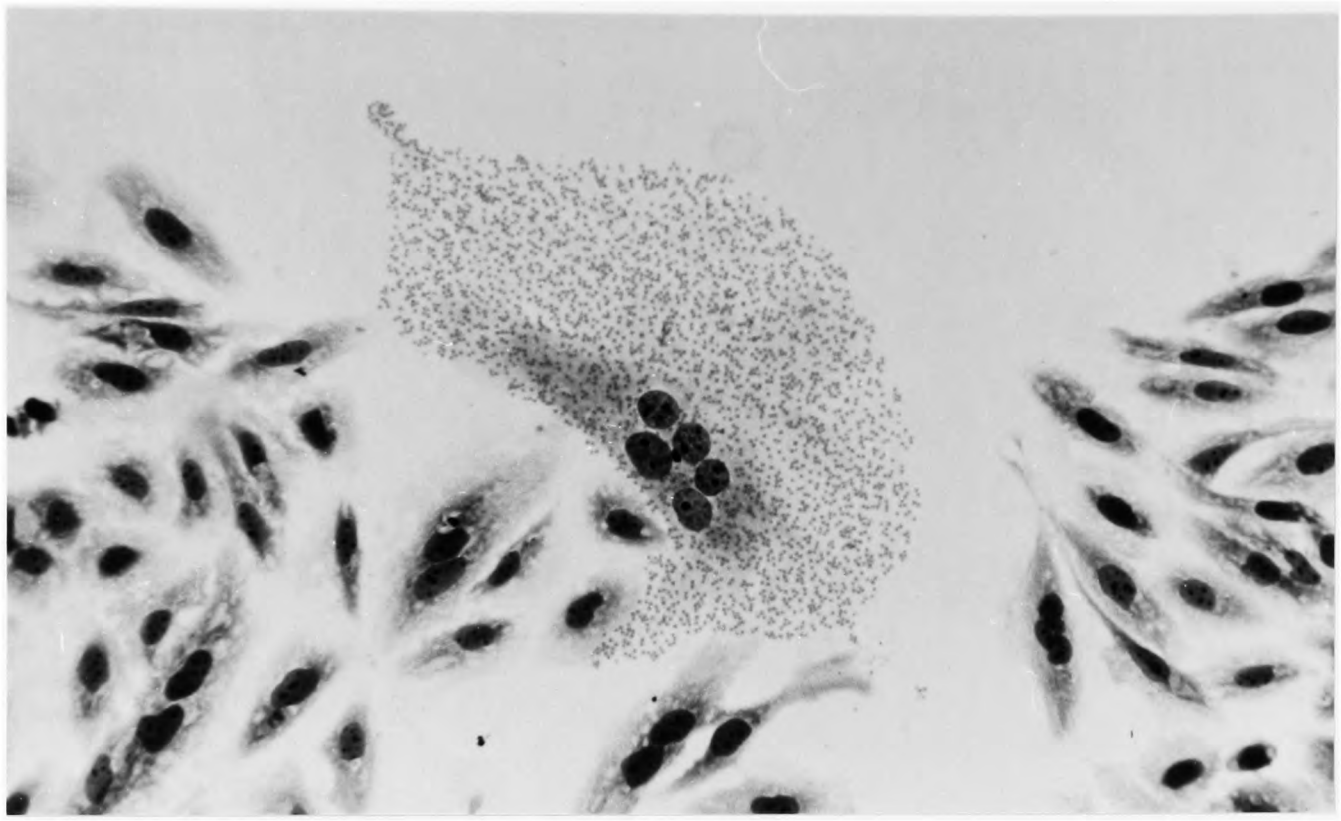

Figure 28. HeLa-Ehrlich heterokaryon 9 days after cell fusion. Labelling for Ehrlich antigens extends far beyond the visible margins of the cell. Many unlabelled HeLa cells are present in the same field. x 130.

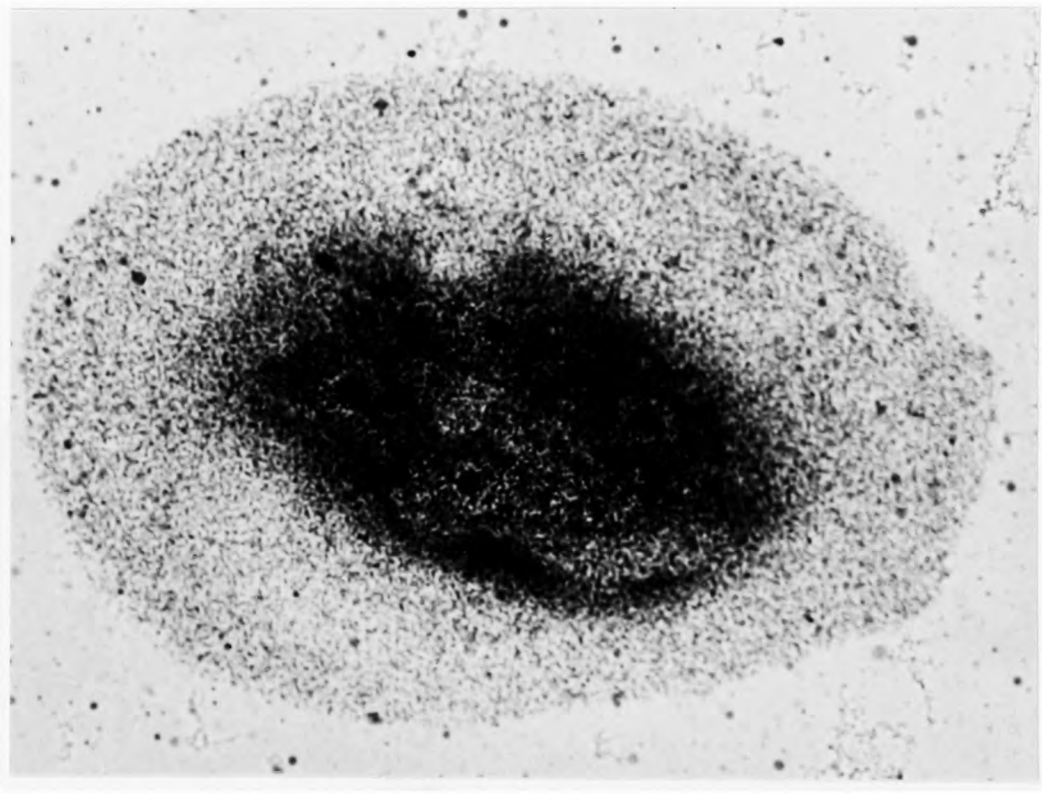

Figure 29. HeLa-Ehrlich heterokaryon 24 hours after fusion. The cell was exposed to [^{3}H]-leucine and labelling for protein synthesis extends beyond the visible cell margins. x 320.

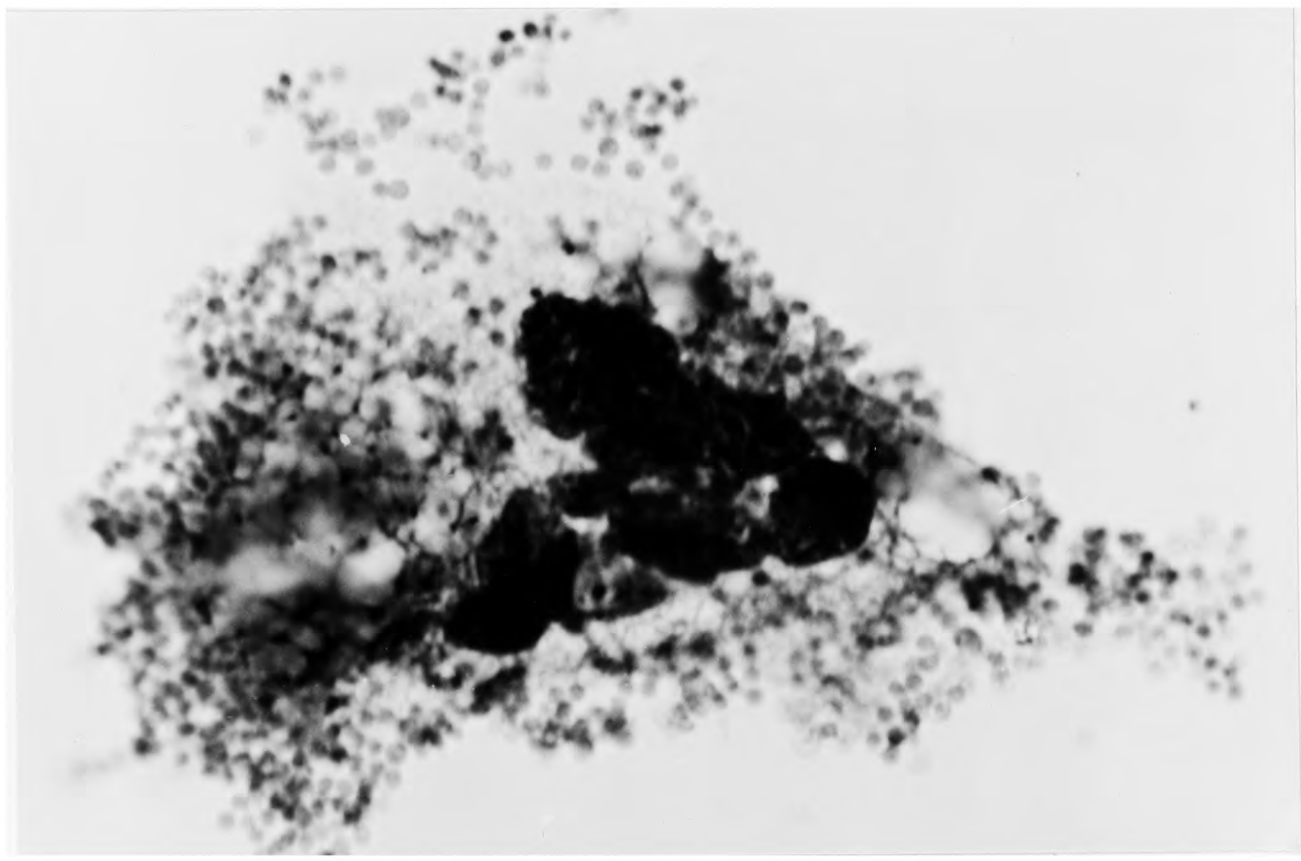

Figure 30. Degenerating HeLa-Ehrlich heterokaryon labelled for Ehrlich antigens 25 days after fusion. x 600.

cells carrying Ehrlich antigens were seen from two or three days after fusion, as shown in Figs. 22 and 23. After 10 days of cultivation, groups containing up to 20 cells were found with Ehrlich surface antigens. Such a group of cells is shown in a fixed and stained preparation 17 days after cell fusion (Fig. 24). The unlabelled cells are HeLa cells. Fig. 25 shows a living preparation in which a group of cells were labelled for Ehrlich antigens by mixed haemadsorption while growing on the floor of a plastic Petri dish. There are unlabelled HeLa cells in the same field.

One unusual feature of these heterokaryons was the fibroblastic appearance as seen in Fig. 23, a mode of growth which differed from that of either parent. Many peculiar cytoplasmic projections were seen, as illustrated in Figs. 26 and 27, and these were labelled for the antigens of each parent. Some large heterokaryons showed antigenic labelling well beyond the visible margins of the cell (Fig. 28) but this material was probably an integral part of the cell since it was rarely detected after the heterokaryons came off the coverslip. Thus it must be differentiated from the discrete areas of antigenic material left on glass after the breaking of local adhesions by single cells (Weiss and Lachmann, 1964). Such a surface zone was also seen about heterokaryons heavily labelled with [^{3}H] leucine (Fig. 29).

The presence of Ehrlich antigen was detected on heterokaryons as late as 25 days after cell fusion but by this time the cells were vacuolated and degenerating (Fig. 30).

Heterokaryons, 24 hours after fusion, were also treated for 60 minutes

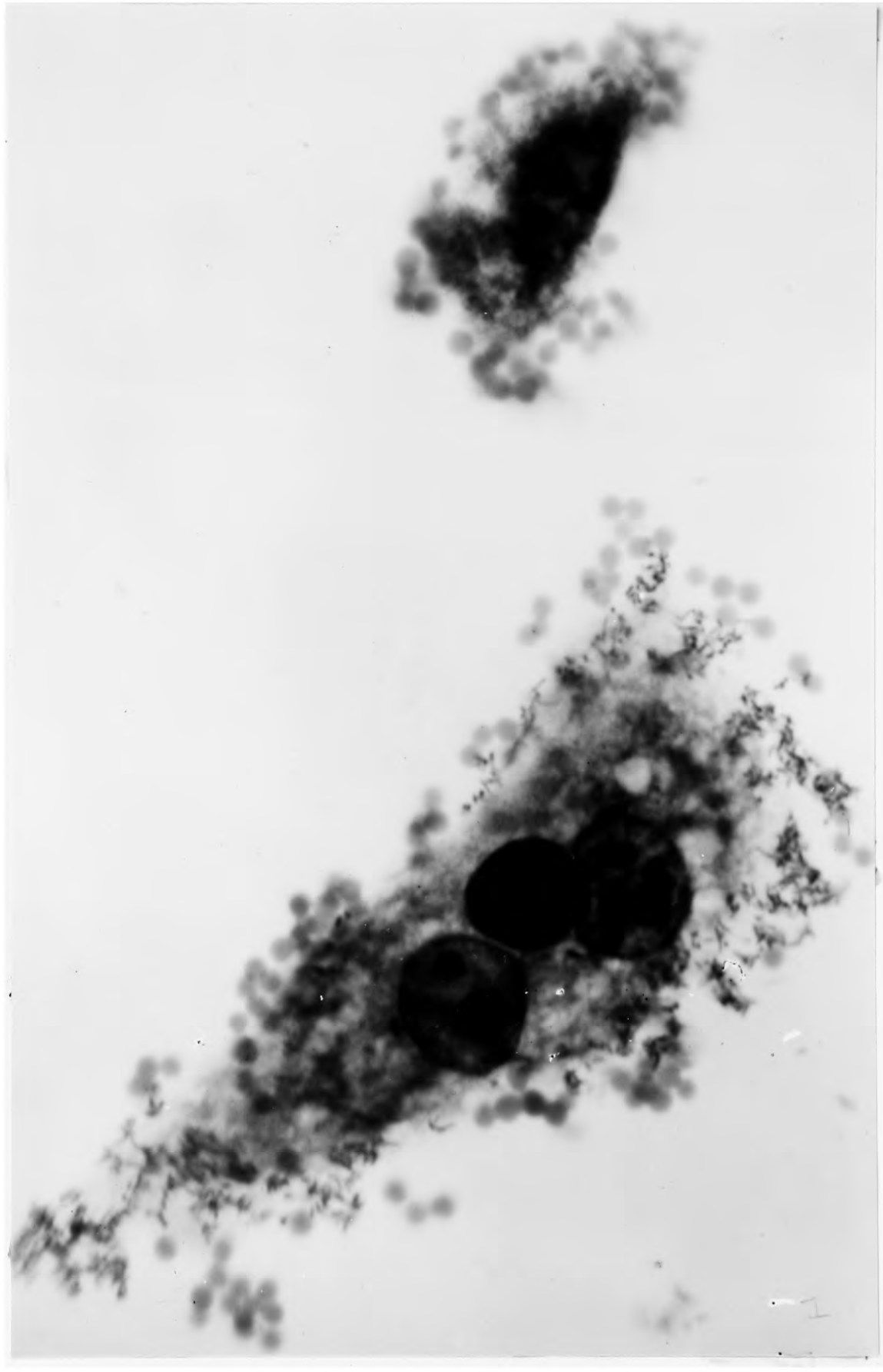

Figure 31. HeLa-Ehrlich heterokaryon labelled for HeLa antigens with erythrocytes and for Ehrlich antigens with bacteria. A single HeLa cell labelled only with erythrocytes is also present. x 1200.

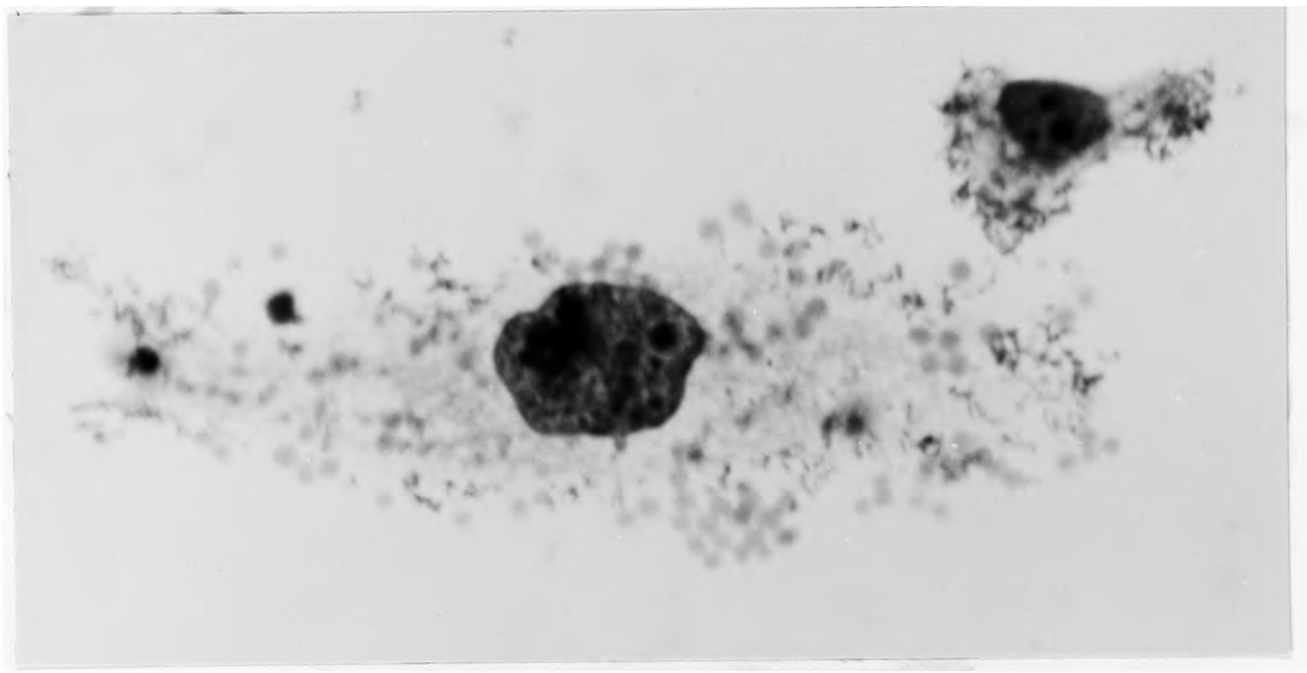

Figure 32. Double labelling on a HeLa-Ehrlich heterosynkaryon 24 hours after cell fusion. In this case rabbit anti-HeLa and guinea pig anti-Ehrlich sera have been used. The single HeLa cell is labelled with bacteria only. x 400.

with neuraminidase at a concentration of 100 units/ml. This did not affect the degree of mixed haemadsorption on HeLa-Ehrlich heterokaryons with anti-Ehrlich serum.

(ii) Double labelling with erythrocytes and bacteria

All multinucleate cells having recognisable Ehrlich nuclei showed double labelling with rabbit anti-Ehrlich and guinea pig anti-HeLa sera (Fig. 31). Some doubly labelled mononucleate cells were also seen within several days of fusion (Fig. 32).

In order to make a more accurate assessment of the ability of HeLa-Ehrlich heterokaryons to multiply some of these cells were cultivated on a feeder layer of gamma-irradiated cells. Approximately 10^7 Ehrlich cells and 2×10^6 HeLa cells were treated with 4,000 HAU of virus and the resulting population of hybrid and HeLa cells was then grown on coverslips for 4 days. The cells on one coverslip were harvested with EDTA, mixed with 5×10^5 HeLa cells which had received 4,000 r of gamma-irradiation and then seeded onto coverslips in a Petri dish containing 5 ml. of medium. Mixed haemadsorption with antiserum against Ehrlich cells was carried out on some comparable coverslips when the cells were harvested. This showed that each coverslip bore, at this time, about 1.4×10^3 single HeLa cells and 2.3×10^3 hybrid cells of which 3.8×10^2 were either dikaryons or hybrid mononucleate cells.

The cells were incubated with the feeder layer for one week with changes of medium every two days. Coverslips, labelled for Ehrlich antigens after 7 days, showed many small groups of haemadsorption-

Figure 33. Clone of fibroblastic HeLa-Ehrlich heterosynkaryons labelled for HeLa antigens with erythrocytes and for Ehrlich antigens with bacteria. x 800.

positive cells amidst larger clones of unlabelled HeLa cells. Double labelling carried out on such coverslips showed that the large clones of cells had HeLa surface antigens only while the smaller clones had both HeLa and Ehrlich surface antigens. Fig. 33 shows a clone of hybrid cells labelled with both bacteria and erythrocytes for HeLa and Ehrlich surface antigens. The cells in these hybrid clones were fibroblastic in appearance and were much more motile than HeLa cells, as revealed by the presence of long cytoplasmic processes and the much more dispersed character of the hybrid clones. The cytoplasm of the hybrid cells frequently showed a vacuolated texture.

From the samples on the coverslips a rough estimate could be made of the cloning efficiency of the HeLa cells and of the hybrid cells. For HeLa cells this was calculated to be about 70% and for all hybrid cells about 10%. If, however, only hybrid cells which were originally dikaryons are capable of any multiplication, the cloning efficiency for these might be as high as 60%. The sampling error involved in selecting coverslips from a Petri dish is large, so that these figures for cloning efficiency are subject to considerable error; but they indicate in any case that a substantial proportion of HeLa-Ehrlich hybrid cells are capable of at least some multiplication.

The largest HeLa clone observed after 7 days cultivation contained about 330 cells, representing between 8 and 9 cell divisions, and the average number of cells per HeLa clone was about 60, representing about 6 cell divisions. The largest hybrid clone at 7 days contained 12 cells, representing between 3 and 4 divisions, and the average number of cells

Table 6

Cytolysis of Cells in Suspension

Cells	Dilution of antiserum	
	Anti-Ehrlich	Anti-HeLa
HeLa	< 1/2	256
Ehrlich	1/1000	<1/2
L	1/1000	<1/2

Table 7

Cytolysis of Cells on a Monolayer

Cells	Dilution of antiserum	
	Anti-Ehrlich	Anti-HeLa
HeLa	< 1/5	1/100
Ehrlich	Not done	Not done
L	1/100	< 1/5

per hybrid clone was 6. Hybrid clones containing more than 20 cells are rarely found even on more prolonged cultivation. This appears to indicate that, under the usual cultural conditions, the growth potential of these hybrid cells is limited. Both HeLa and Ehrlich surface antigens are retained during the process of multiplication.

(iii) Detection of Ehrlich surface antigen one hour after cell fusion

The double labelling experiments indicated that the surface antigens of the two cell species were randomly mixed. It was of interest to determine the speed with which this mixing occurred. For this purpose Ehrlich ascites cells were fused with HeLa cells by settling them onto a preformed monolayer of the latter in the presence of inactivated Sendai virus. It was found that when mixed haemadsorption was performed one hour after fusion by this method, erythrocytes which had adsorbed to the large amounts of virus still present on the surface of the cells eluted slowly and incompletely. Labelling was therefore carried out by treating the monolayer with rabbit versus Ehrlich antiserum followed by doubly sensitised bacteria, which do not adsorb to virus. One hour after fusion many heterokaryons were present, and the bacteria usually adsorbed over the whole surface of such cells. Occasional heterokaryons were also seen in which labelling was confined to the area of cell surface overlying the Ehrlich nucleus.

(iv) Cytolysis

The cytolytic titre of anti-HeLa and anti-Ehrlich sera is shown in Table 6. It was of interest that anti-Ehrlich serum did not lyse HeLa cells and anti-HeLa serum did not lyse Ehrlich cells although

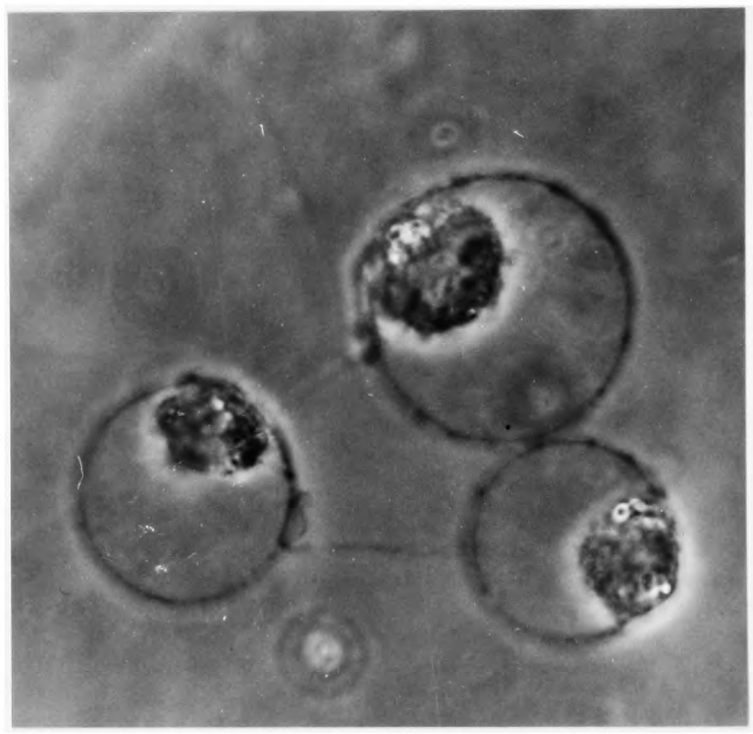

Figure 34. Cytolysis of suspended Ehrlich ascites cells with anti-Ehrlich serum and guinea pig complement. x 600.

marked cross-reaction occurred in mixed haemadsorption experiments with unabsorbed antiserum. The reason for titrating anti-Ehrlich serum with L cells will be discussed below. The effect of anti-Ehrlich serum on suspended Ehrlich cells is shown in Fig. 34.

It is difficult to obtain a definite end-point for the cytolysis of monolayer cells which are subsequently stained, yet cytolysis must be done in this way so that nuclei can be identified and heterokaryons differentiated from homokaryons. Identification of heterokaryons in suspension is virtually impossible and more quantitative methods such as the release of radioactive labels from lysed cells (Klein and Perlmann, 1963; Sanderson, 1965) are not possible because of the presence of single unfused cells. Lysed cells on a monolayer go through a sequence of morphological changes beginning with cytoplasmic swelling and irregularity, nuclear swelling, and prominence of the nuclear membrane. The final product in stained preparations is a 'shattered' cell with a pyknotic nucleus and disorganised cytoplasm. Reduction in the number of adherent cells is usual after antiserum treatment but some severely lysed cells may remain adherent to the coverslip. The end-point chosen here was a marked morphological change with nuclear pyknosis in all monolayer cells. It is realised that this is not an extremely precise measurement.

One difficulty in titration of antisera was that the strain of Ehrlich cells used did not grow on a monolayer. Yet it was desirable to use these cells because their nuclei, unlike those of L cells, are clearly distinguishable from HeLa nuclei, and because of the previous work

Table 8

Survival of Heterokaryons in Antiserum and Complement

Experiment number	serum concentration	Normal rabbit serum	Anti-Ehrlich serum	Anti-HeLa serum
1	1/5	7.0×10^2	1.5×10^2	0
	1/20	not done	3.5×10^2	2
	1/100	1.3×10^3	1.2×10^3	1.0×10^2
2	1/2	1.1×10^3	0	0
	1/10	1.4×10^3	0	0
	1/50	1.0×10^3	9.0×10^2	0
3	1/10	4.8×10^3	3.0×10^2	not done
	1/50	6.6×10^3	2.9×10^3	not done
4	1/5	6.1×10^3	1.8×10^3	0
	1/20	5.3×10^3	not done	0
	1/100	3.5×10^3	not done	0
5	1/2	6.0×10^2	0	0
	1/10	1.8×10^3	5.0×10^2	0
	1/20	1.9×10^3	5.5×10^2	0
6	1/5	2.5×10^3	1.5×10^3	not done
	1/20	2.8×10^3	7.5×10^2	not done
	1/100	1.5×10^3	1.8×10^3	not done

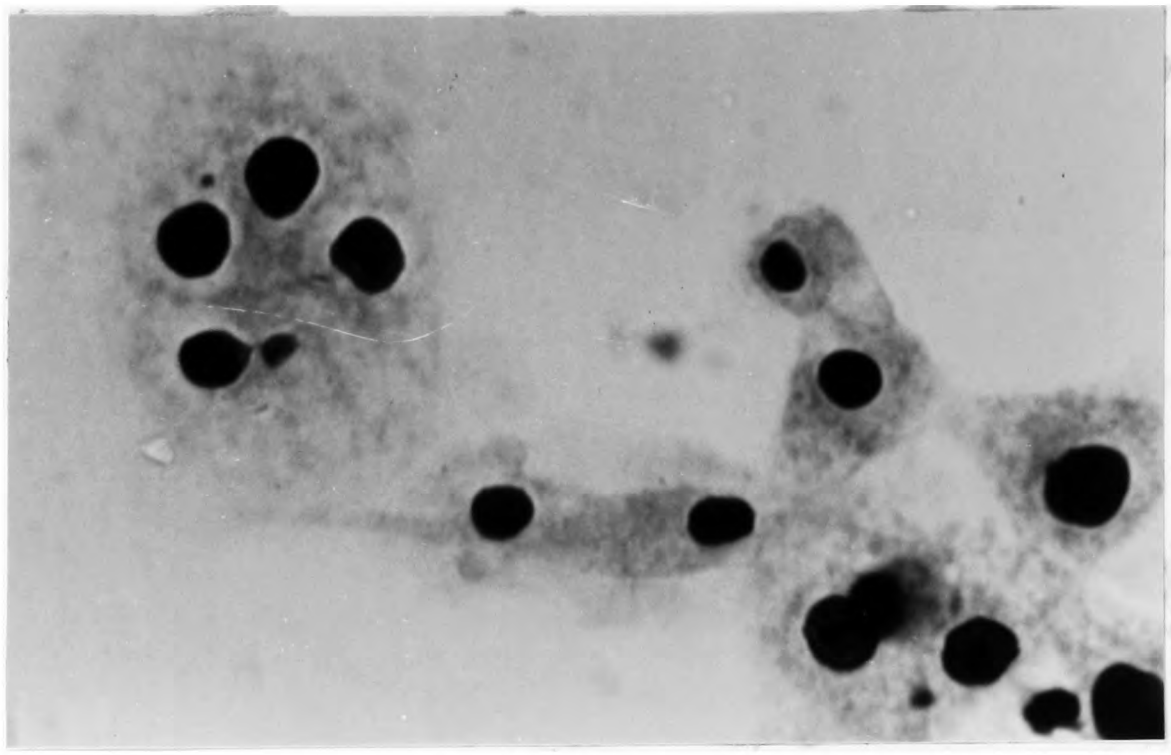

Figure 35. HeLa-Ehrlich heterokaryon and single HeLa cells lysed on a monolayer by 1/20 anti-HeLa serum and guinea pig complement. x 600.

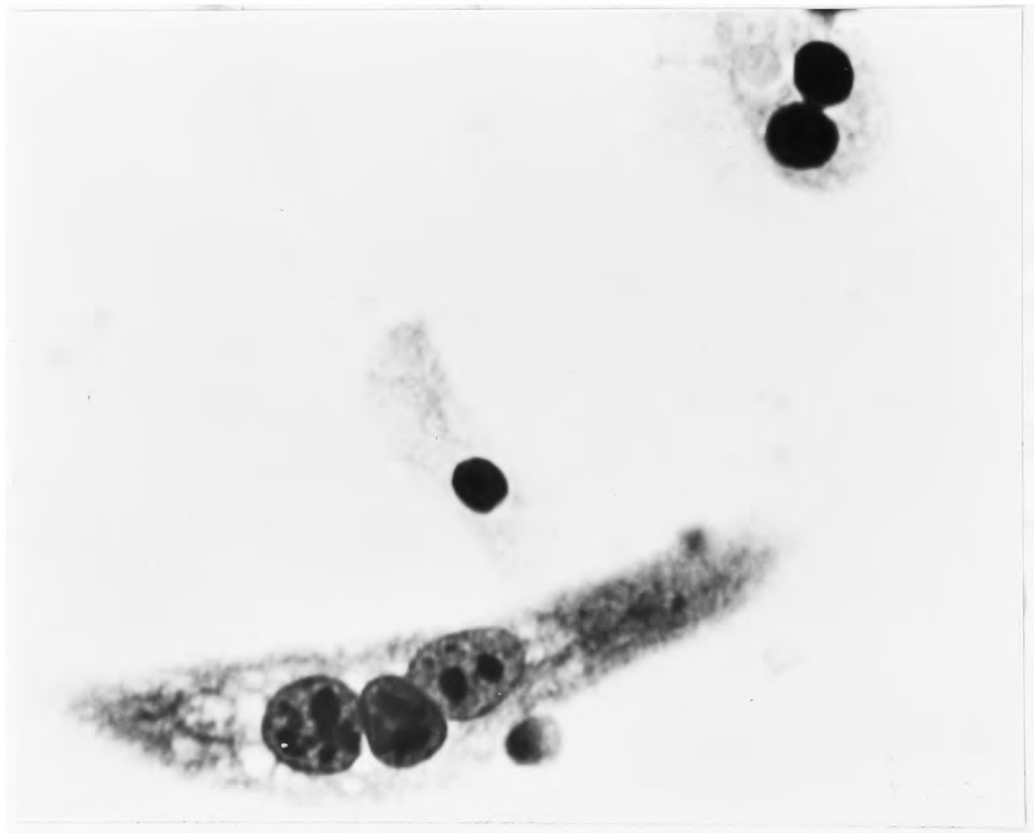

Figure 36. HeLa-Ehrlich heterokaryon , containing one HeLa and two Ehrlich nuclei, one day after fusion. It is unlysed by 1/20 anti-HeLa serum and guinea pig complement. A HeLa homokaryon and a single HeLa cell in the same field have been lysed. x 600.

carried out on the surface antigens of HeLa-Ehrlich heterokaryons. Cytolysis on monolayers was therefore carried out with L cells. As shown in Table 6, AES lysed Ehrlich cells, and L cells of mouse origin, to the same extent in suspension. Table 7 shows cytolysis on monolayers. It is noted that a higher concentration of antiserum was needed to produce morphological changes in 2 hours on a monolayer than was required for cells in suspension.

An initial experiment was done to assess whether multinucleate cells were more resistant to cytolysis than single cells. 4,000 HAU of inactivated Sendai virus was added to 5×10^6 HeLa cells in suspension. Cytolysis of the resulting homokaryons with anti-HeLa serum revealed that they were as susceptible to antiserum as single HeLa cells as long as the monolayers were of comparable density. Confluent monolayers, whether of single cells or homokaryons, were more resistant to cytolysis than widely dispersed cells.

For heterokaryon experiments 2×10^6 HeLa cells were fused with 10^7 Ehrlich ascites cells using 4,000 HAU of virus. The results of cytolysis experiments are summarised in Table 8, which shows the number of surviving heterokaryons per coverslip after exposure for 2 hours to antiserum and 1/20 guinea pig complement. There was some variation in results from experiment to experiment. Heterokaryons were almost as susceptible to anti-HeLa serum as single HeLa cells although occasional heterokaryons were resistant to concentrations of antiserum which lysed adjacent single HeLa cells (Figs. 35 and 36). Results with anti-Ehrlich serum varied from 100% lysis with 1/10 antiserum in experiment 2 to 40% lysis

Table 9

Cytolysis of HeLa-Ehrlich Heterokaryons with a Combination of Antisera

Serum	Concentration	Number of unlysed heterokaryons
Normal rabbit	1/5	6.1×10^3
Normal rabbit	1/100	3.5×10^3
Anti-Ehrlich	1/5	1.0×10^3
Anti-HeLa	1/500	2.2×10^3
Anti-Ehrlich and anti-HeLa	1/5 1/500	0

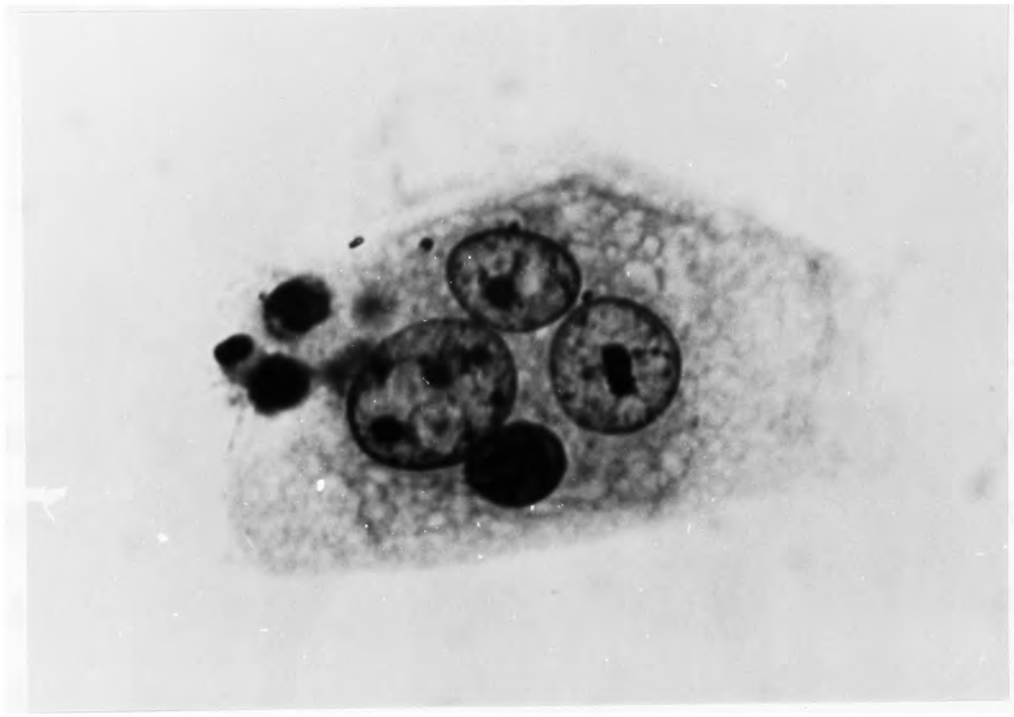

Figure 37. HeLa-Ehrlich heterokaryon after 2 hours of exposure to 1/20 anti-HeLa serum and guinea pig complement. One Ehrlich and two HeLa nuclei show considerable osmotic swelling while one Ehrlich nucleus has retained a fairly normal morphology. x 600.

in experiment 6. In general heterokaryons grown in foetal calf serum medium (experiments 4-6) flattened out better, appeared healthier, and were more resistant to cytolysis than those grown in calf serum medium (experiments 1-3).

It was of interest to see if heterokaryons could be lysed by combinations of antisera in concentrations at which single sera were not completely effective. The results of such an experiment are shown in Table 9. There is summation of activity of the two antisera.

In some experiments a considerable difference in the degree of osmotic swelling of nuclei in partially lysed heterokaryons was noted (Fig. 37).

Because of the overgrowth of heterokaryon cultures by single HeLa cells and because of the disappearance of some heterokaryons and nuclear fusion in others, meaningful cytolysis experiments could not be carried out some days after the fusion of normal HeLa and Ehrlich cells. Cytolysis was done, however, some days after fusion of normal Ehrlich cells with gamma-irradiated HeLa cells and will be described in the next section.

In one experiment heterokaryons produced in the usual way were allowed 24 hours to attach to coverslips. They were grown a further 24 hours in medium containing 1/100 anti-Ehrlich serum and then stained. Heterokaryons showed no morphological changes but there was a definite inhibition of nuclear fusion.

(v) Gamma-irradiation of HeLa cells before fusion

After receiving 8,000 r virtually all HeLa cells came off the glass in 4 or 5 days. However, heterokaryons adhered to glass for a much

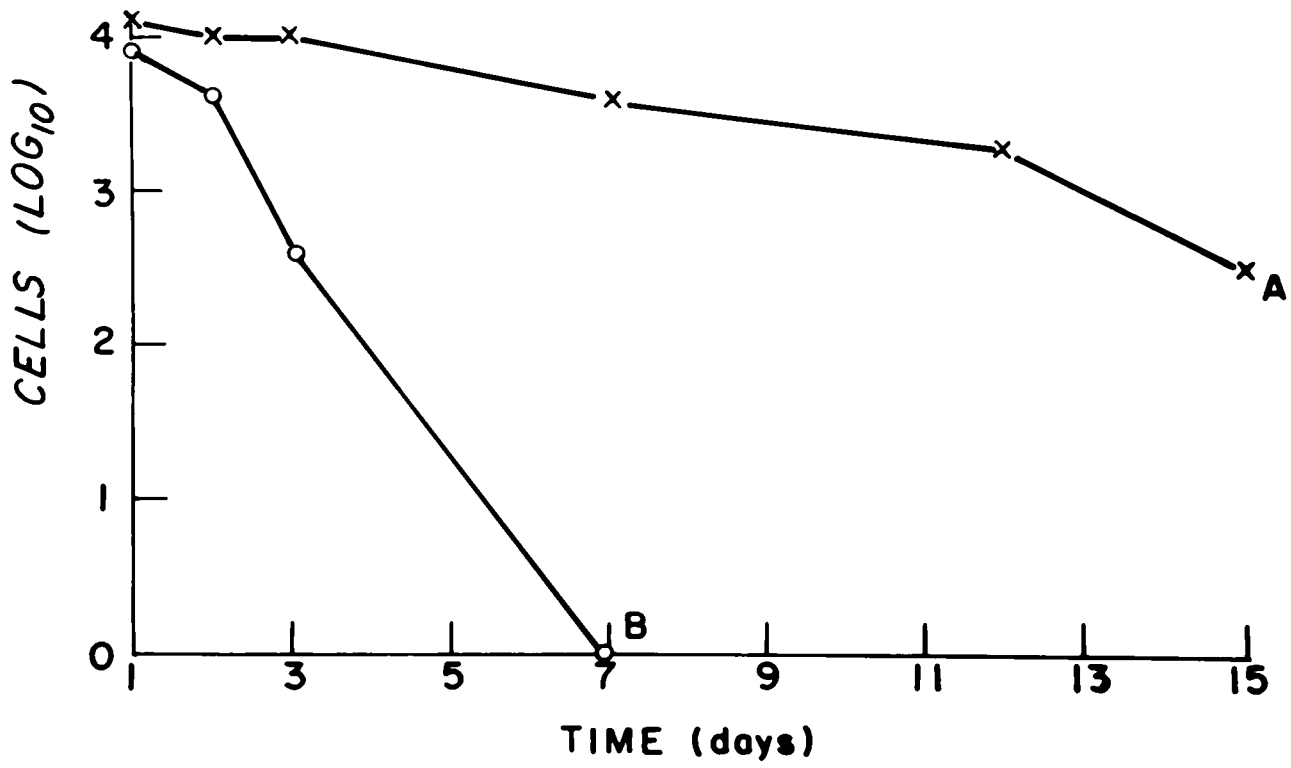

Figure 38. Persistence of cells after fusion of gamma-irradiated HeLa cells (8,000 r) with unirradiated Ehrlich cells. A represents all cells labelled for Ehrlich antigens (heterokaryons) and B all cells unlabelled for Ehrlich antigens (single HeLa cells and HeLa homokaryons).

Table 10

Cytolysis of Heterokaryons after Fusion of Gamma-irradiated HeLa Cells with Normal Ehrlich Cells

Serum	Dilution	4 days after fusion	7 days after fusion
Normal rabbit	1/5	4.4×10^3	2.3×10^3
Anti-Ehrlich	1/5	1.7×10^3	1.1×10^3
Anti-HeLa	1/5	1.0×10^2	25
Anti-HeLa	1/100	4.1×10^2	5.5×10^2
Anti-Ehrlich and anti-HeLa	1/5 1/100	0	0

longer period when 3×10^6 gamma-irradiated HeLa cells were fused with 10^7 normal Ehrlich cells. Fig. 38 shows the results of such an experiment. Single HeLa cells and HeLa homokaryons were completely gone 7 days after fusion. Many cells labelled for Ehrlich antigens were still present 2 weeks after fusion although many of these appeared vacuolated and unhealthy by this time. All these cells were also labelled for HeLa antigens. Considerably more nuclear fusion occurred in the heterokaryons than in HeLa homokaryons. When single Ehrlich cells were simply mixed with gamma-irradiated HeLa cells, the Ehrlich cells did not adhere to glass and no clones labelled for Ehrlich antigen appeared.

Cytolysis was carried out on the heterokaryons produced with gamma-irradiated HeLa cells and normal Ehrlich cells 4 and 7 days after fusion. The results are summarised in Table 10, which shows the number of heterokaryons per coverslip after 2 hours of exposure to antiserum and 1/20 guinea pig complement at $37^{\circ}C$. Mixed haemadsorption showed that all cells were labelled for both HeLa and Ehrlich antigens at this stage so all cells could be counted regardless of the degree of nuclear fusion. Heterokaryons were still more resistant to anti-Ehrlich serum than to anti-HeLa serum and there was still summation of activity with combinations of antisera.

When both HeLa and Ehrlich cells were irradiated before cell fusion the resultant heterokaryons also adhered to coverslips for a much longer time than irradiated HeLa homokaryons.

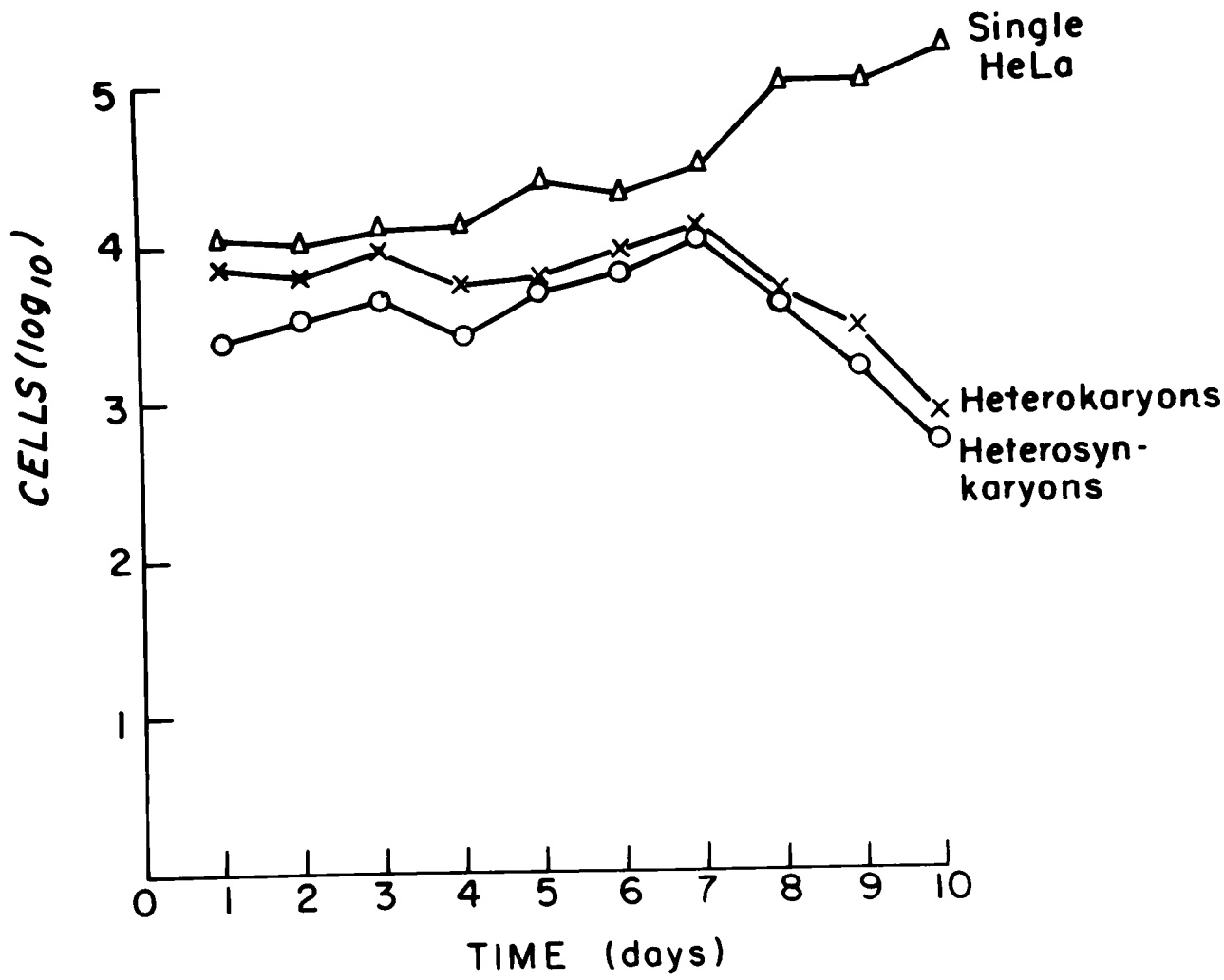

Figure 39. Number of single HeLa cells, heterokaryons, and heterosynkaryons after the fusion of 2×10^6 HeLa cells with 10^7 normal Ehrlich cells.

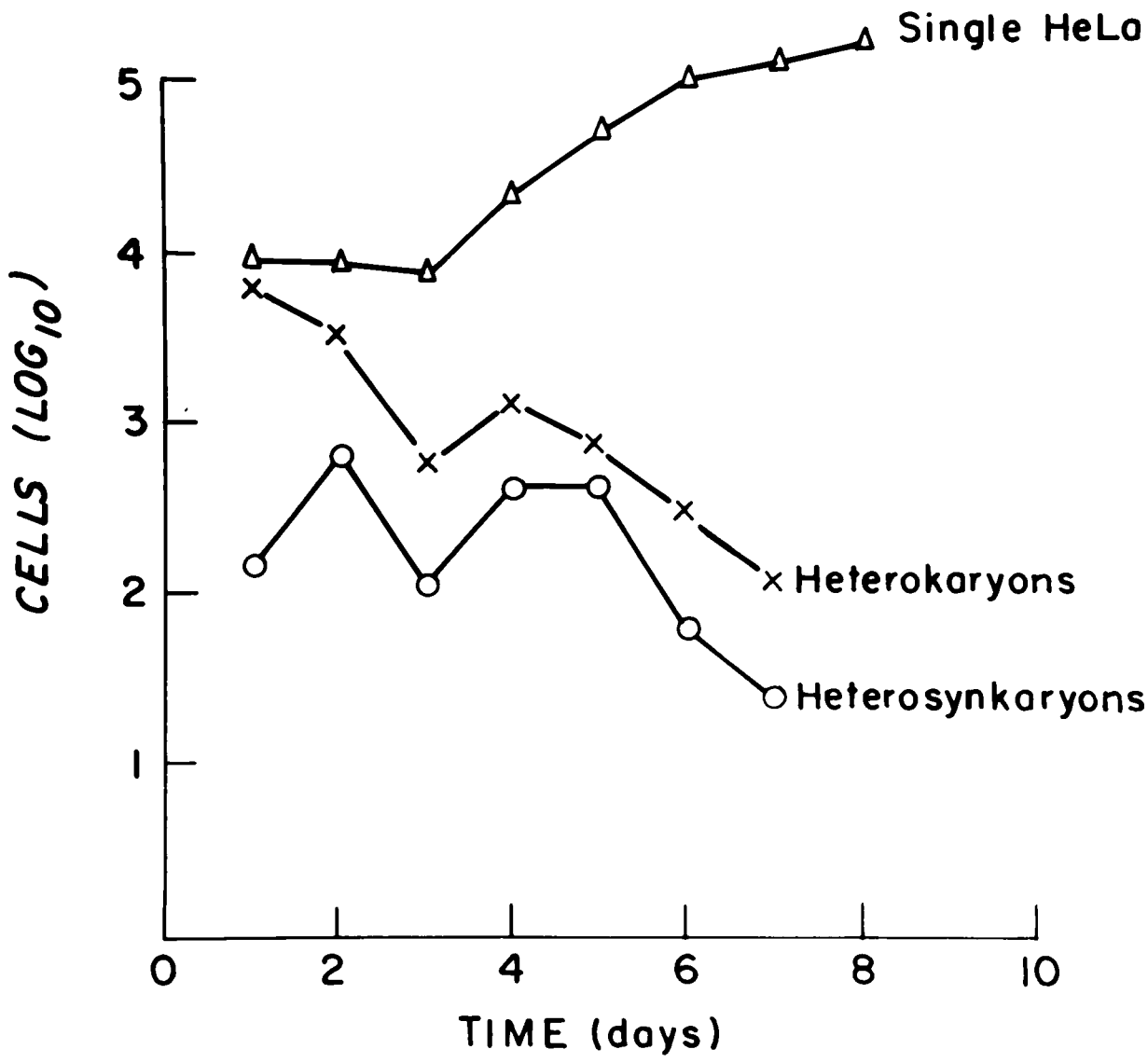

Figure 40. Number of single HeLa cells, heterokaryons, and heterosynkaryons after the fusion of 2 x 10^6 HeLa cells with 10^7 UV-irradiated Ehrlich cells (3,000 ergs/mm^2).

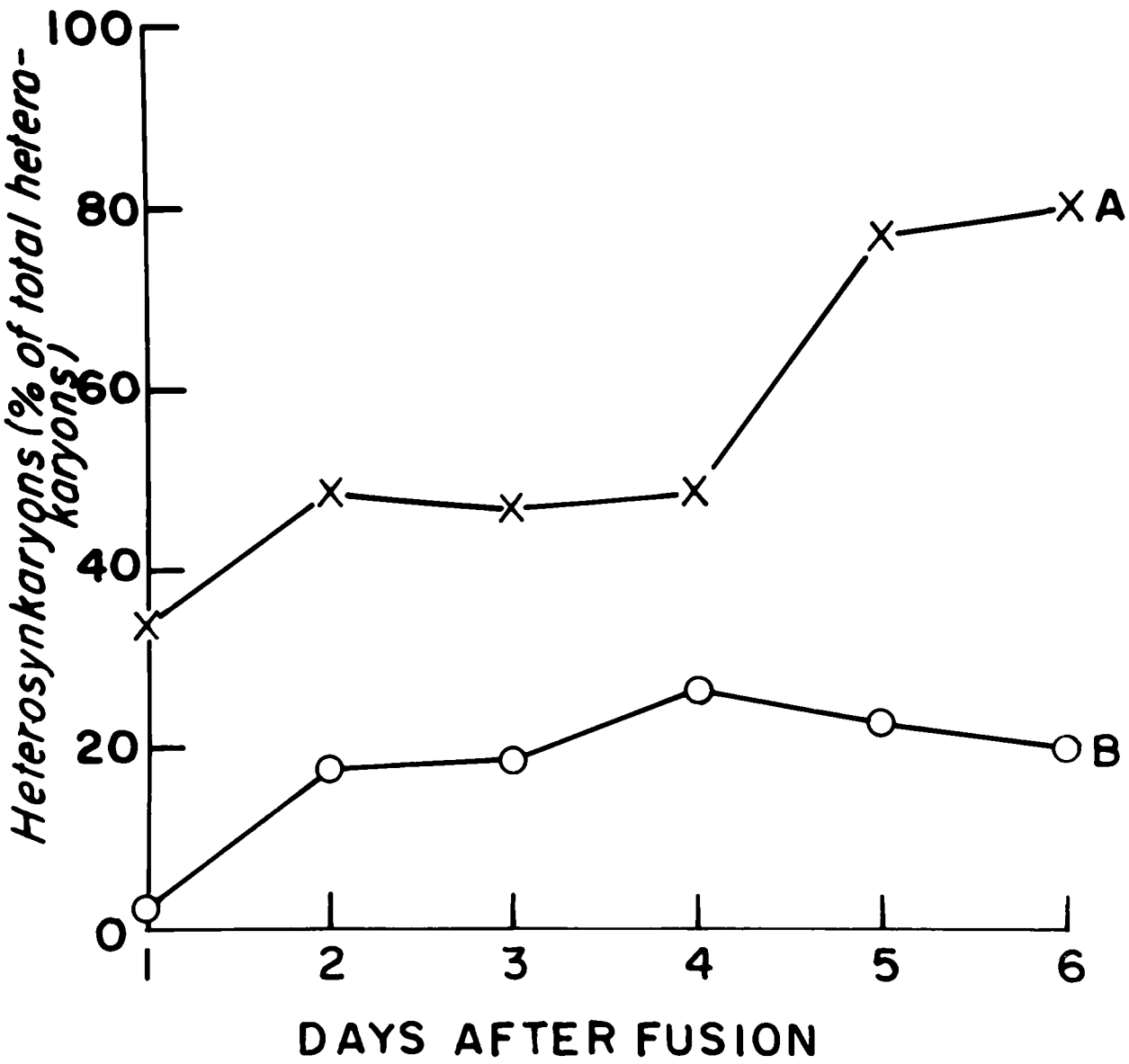

Figure 41. Percentage of heterokaryons which are heterosynkaryons after fusion of (A) normal HeLa cells and Ehrlich cells and (B) normal HeLa cells and UV-irradiated Ehrlich cells.

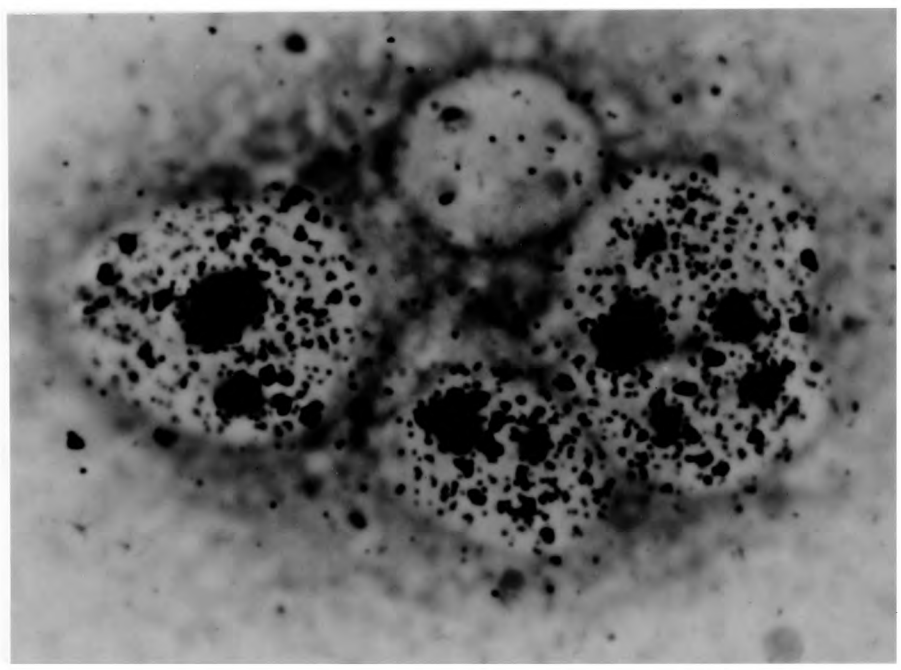

Figure 42. Autoradiograph showing RNA synthesis in an Ehrlich-HeLa heterokaryon 24 hours after fusion. Ehrlich cells were UV-irradiated before fusion and the single Ehrlich nucleus (upper) is not synthesizing RNA. x 1500.

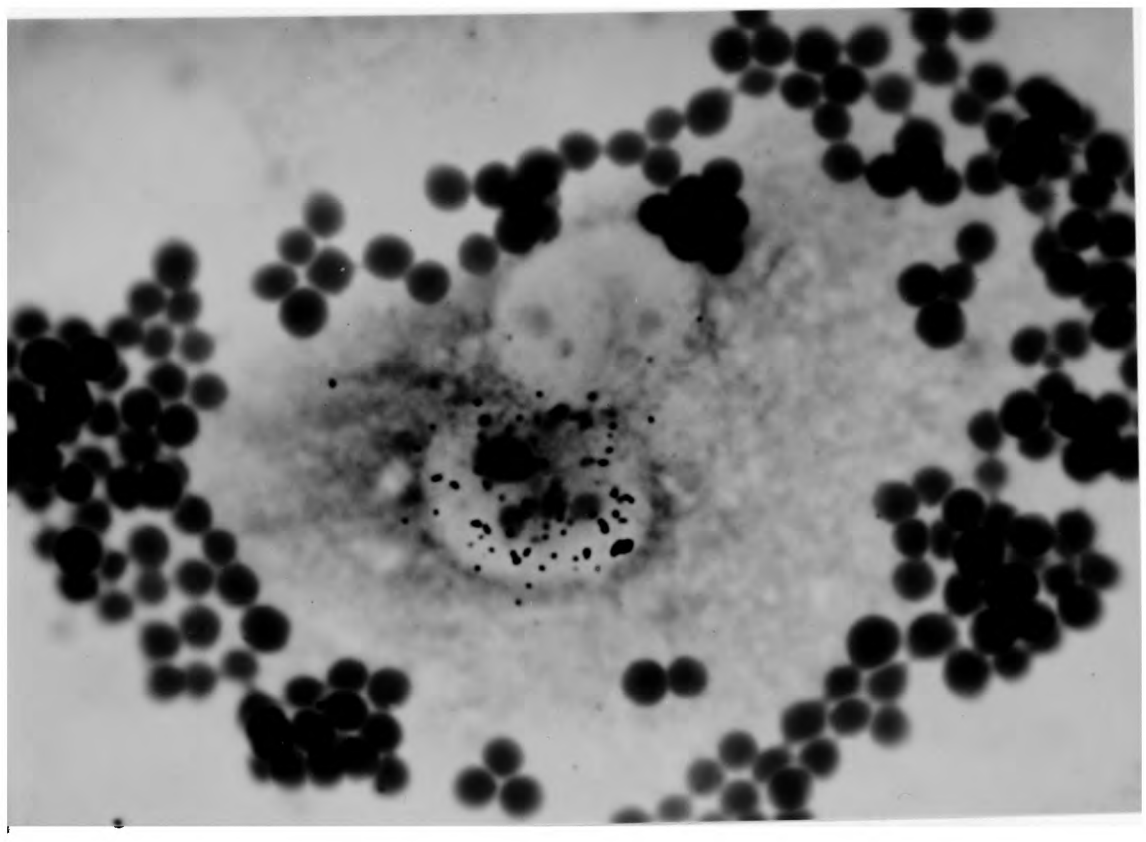

Figure 43. Autoradiograph of a HeLa-Ehrlich heterokaryon 48 hours after cell fusion. The UV-irradiated Ehrlich nucleus is not synthesizing RNA but the cell is strongly labelled for Ehrlich antigens by mixed haemadsorption. x 1200.

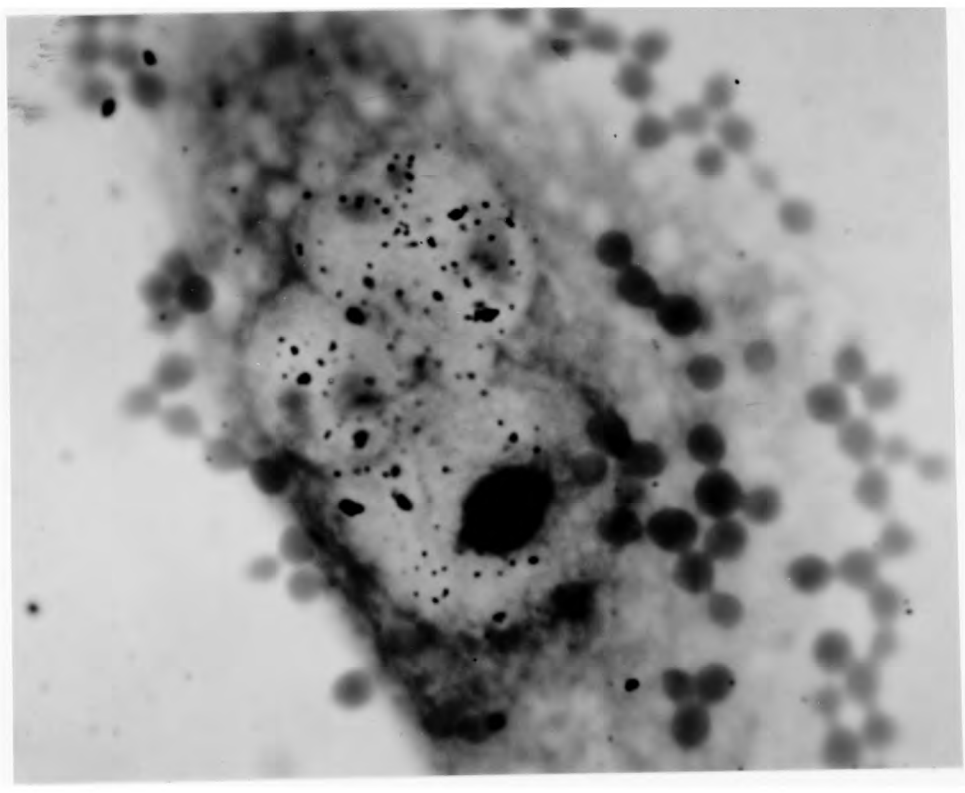

Figure 44. Autoradiograph of a HeLa-Ehrlich heterokaryon 24 hours after fusion. The HeLa nucleus (bottom) and the two Ehrlich nuclei are all synthesizing RNA and the cell is labelled for Ehrlich antigens by mixed haemadsorption. x 1500.

(vi) UV-irradiation of Ehrlich cells before fusion

Heterokaryons produced by fusing normal HeLa and Ehrlich cells carried each species antigens for the life of the cells, a maximum of 2 or 3 weeks. Although some multiplication of hybrid cells did occur, it was still not possible to say whether surface antigens had simply persisted from the time of fusion or whether continued metabolic activity of each component of the heterokaryon was necessary to maintain and replace each antigen on the cell surface. UV-irradiation of Ehrlich cells before fusion was used in an attempt to answer this question.

Figs. 39 and 40 illustrate the effect of a low dose of UV-irradiation on the survival of HeLa-Ehrlich heterokaryons. These heterokaryons died more rapidly than those produced with healthy Ehrlich cells. Fig. 41 shows that UV-irradiation of Ehrlich cells also inhibited nuclear fusion in heterokaryons. In spite of these effects, Ehrlich antigen was still detected on most heterokaryons until they left the glass. Several days after fusion a few heterokaryons were not labelled for Ehrlich antigen but the proportion was variable and never greater than 5%. No unlabelled heterokaryons were seen in a control fusion of normal HeLa and Ehrlich cells.

Autoradiographs confirmed the fact that UV-irradiation prevented RNA synthesis by Ehrlich nuclei in heterokaryons (Fig. 42). However, these cells remained labelled for Ehrlich antigen (Fig. 43) in the same way as heterokaryons in which Ehrlich nuclei were synthesising RNA (Fig. 44). In control cultures mononuclear cells labelled for Ehrlich

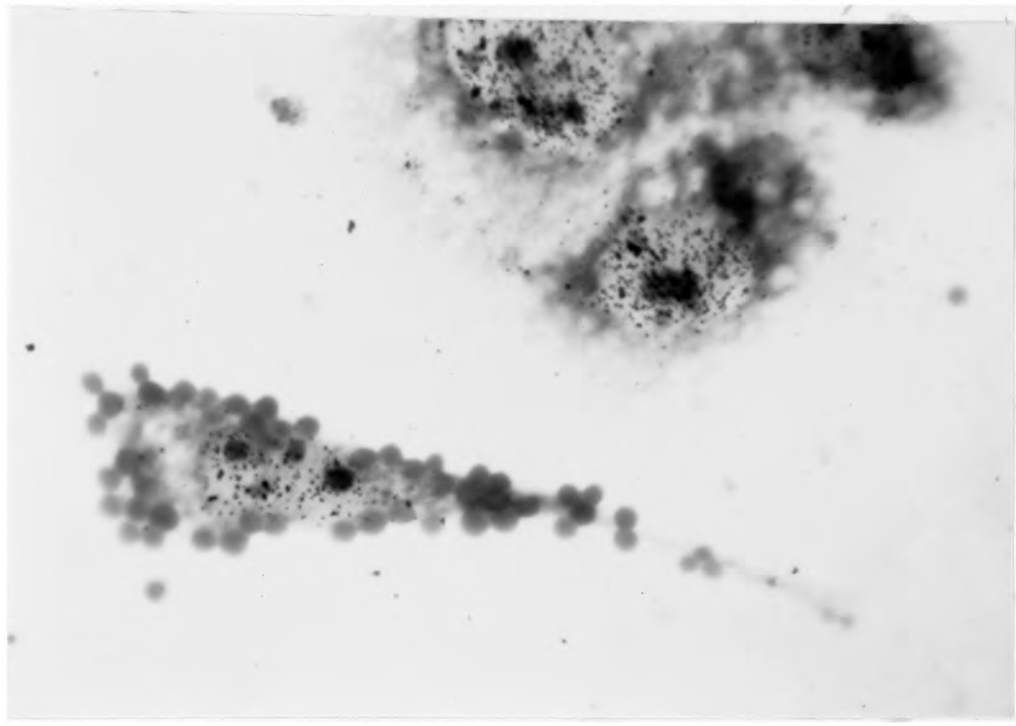

Figure 45. Autoradiograph showing RNA synthesis by HeLa cells and by a HeLa-Ehrlich heterosynkaryon labelled with erythrocytes for Ehrlich antigens. x 720.

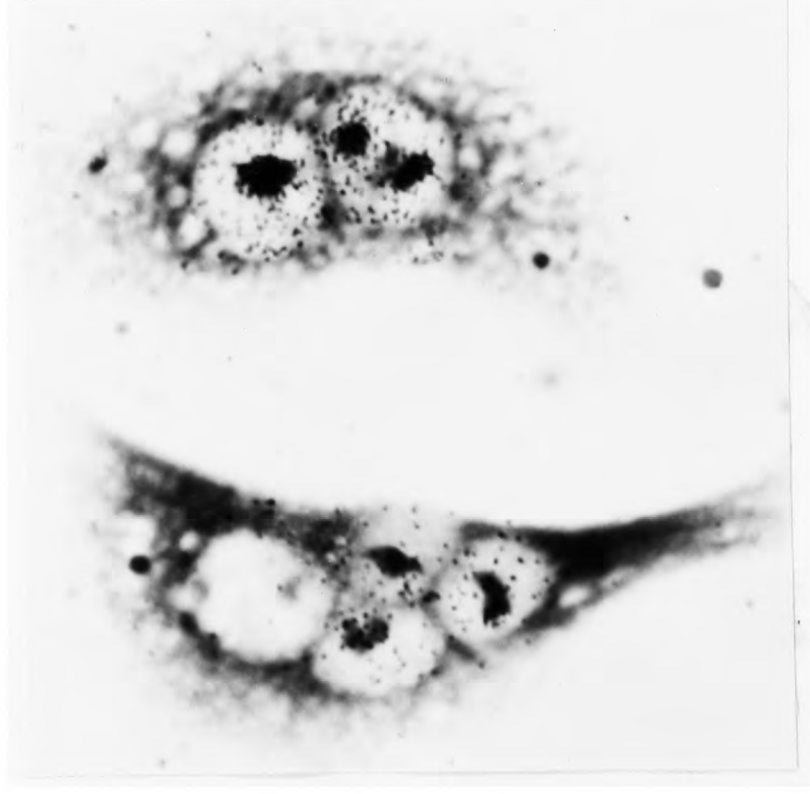

Figure 46. Autoradiograph 24 hours after the fusion of HeLa cells with UV-irradiated Ehrlich cells. HeLa nuclei in the heterokaryon (lower) are not synthesizing RNA as well as HeLa nuclei in the homokaryon. x 720.

antigen continued to show active RNA synthesis (Fig. 45). Often HeLa nuclei in a heterokaryon with a UV-irradiated Ehrlich nucleus did not show as active RNA synthesis as HeLa nuclei in adjacent homokaryons (Fig. 46).

Cytolysis experiments suggested that heterokaryons produced with UV-irradiated Ehrlich cells were slightly more resistant to the cytolytic effect of anti-Ehrlich serum and complement than control heterokaryons. The results, however, were variable. An experiment was therefore set up to compare the antibody response of rabbits to normal Ehrlich cells, HeLa-normal Ehrlich heterokaryons, and HeLa-UV Ehrlich heterokaryons. Weekly fusions of 4×10^6 HeLa cells with 2×10^7 normal Ehrlich cells and with 2×10^7 Ehrlich cells given 30,000 ergs/mm^2 were carried out with 4,000 HAU of virus. Some heterokaryons were grown in medical flat bottles for 24 hours and others in Petri dishes containing coverslips. Twenty-four hours after fusion stained coverslips were examined and the number of heterokaryons and proportion of Ehrlich nuclei determined. Three kilogram rabbits were injected intravenously every week as follows:

Rabbit 1 - 2×10^6 Ehrlich cells

Rabbit 2 - 1.0×10^6 HeLa-normal Ehrlich heterokaryons with an average of 2 Ehrlich nuclei per heterokaryon. There were about 10^6 HeLa cells in the same population.

Rabbit 3 - 1.0×10^6 HeLa-UV Ehrlich heterokaryons with an average of 2 Ehrlich nuclei per heterokaryon. About 1.5×10^6 HeLa cells were also injected.

One week after the second injection a serum sample was taken from

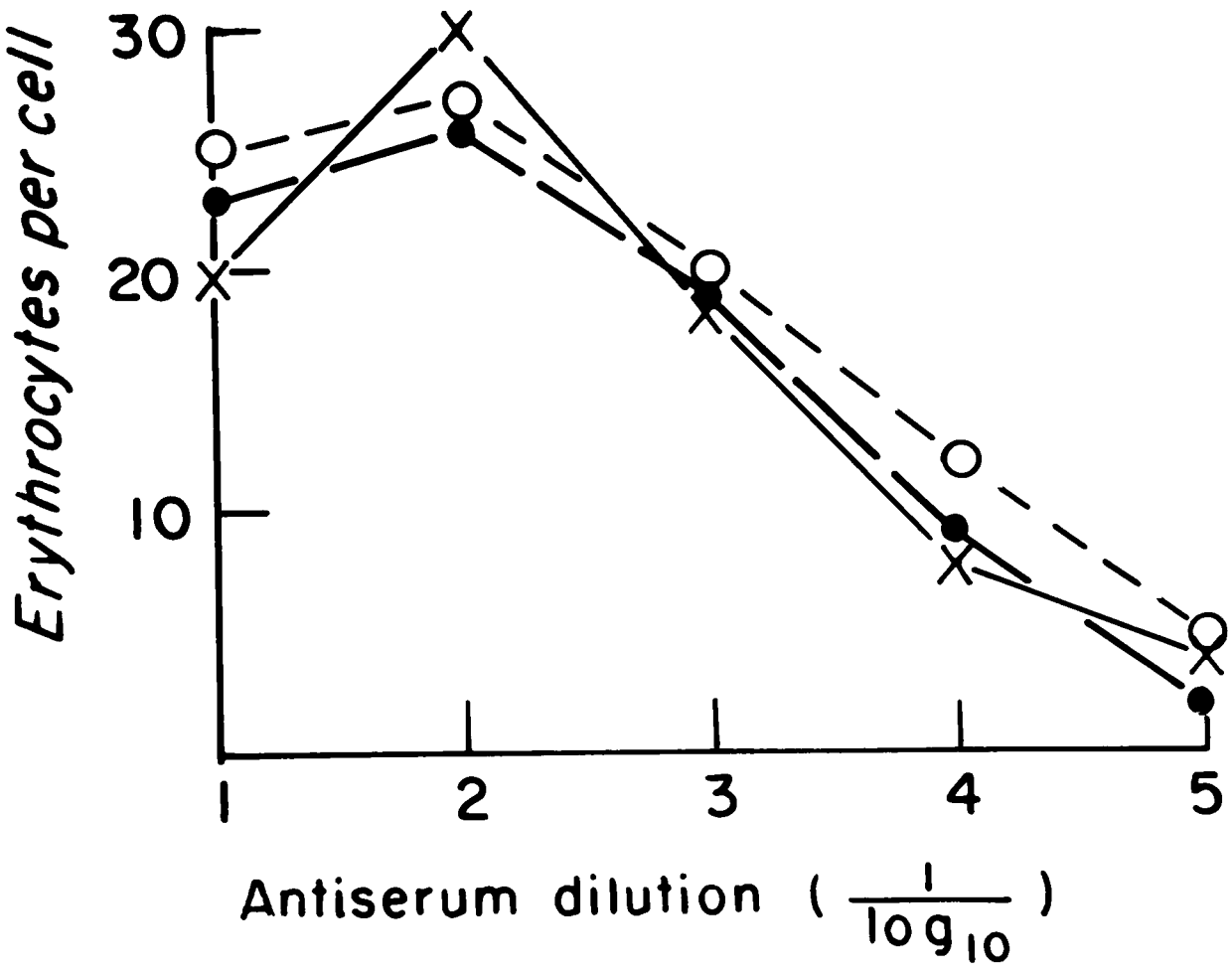

Figure 47. Mixed haemadsorption on L cells with rabbit antiserum to Ehrlich cells, HeLa-normal Ehrlich heterokaryons, and HeLa-UV Ehrlich heterokaryons, one week after the second injection.

Table 11

Cytolysis of Ehrlich Cells

Rabbit number	Cells injected	Serum titre after:	
		2 injections	3 injections
1	Ehrlich	1,000	4,000
2	HeLa-normal Ehrlich heterokaryons	256	4,000
3	HeLa-UV Ehrlich heterokaryons	256	4,000

Table 12

Fusion of Gamma-irradiated HeLa Cells with UV-irradiated Ehrlich Cells

UV dose ($ergs/mm^2$)	Cells per coverslip		
	Single HeLa	HeLa homokaryons	Heterokaryons
0	7.3×10^3	1.2×10^3	1.3×10^4
10,000	7.7×10^3	1.8×10^3	4.5×10^3
30,000	6.2×10^3	1.1×10^3	2.6×10^3

Table 13

Mixed Haemadsorption for Ehrlich Antigen on Heterokaryons

UV dose to Ehrlich cells ($ergs/mm^2$)	Haemadsorption +ve heterokaryons (%)		
	1 day after fusion	2 days after fusion	3 days after fusion
0	100	100	100
10,000	100	84	84
30,000	100	63	68

Table 14

Survival of Heterokaryons after Actinomycin Treatment of Ehrlich Cells

Days after fusion	Single HeLa cells	Heterokaryons
1	7.7×10^3	1.1×10^4
2	1.0×10^3	5.3×10^3
3	50	1.6×10^3
4	0	0

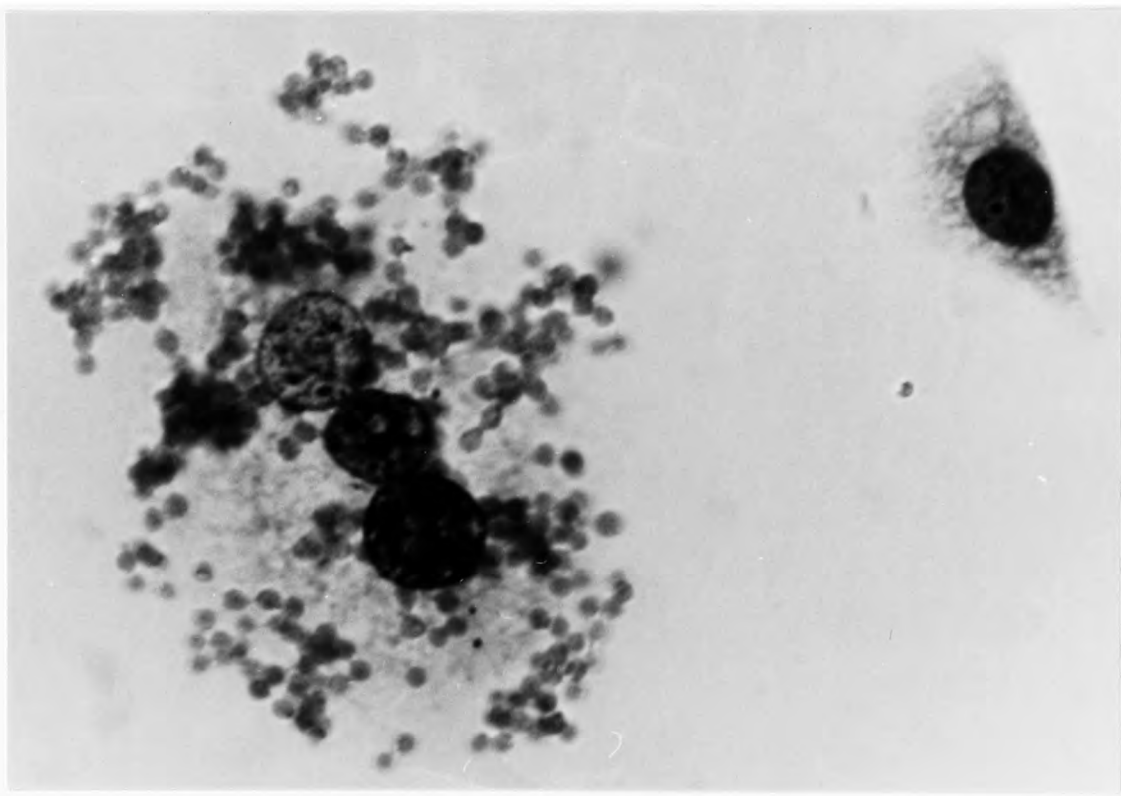

Figure 48. Mixed haemadsorption for Ehrlich antigens on HeLa-Ehrlich heterokaryon 2 days after fusion. Ehrlich cells were treated with actinomycin D before fusion. x 600.

each rabbit and titrated by mixed haemadsorption. The results are shown in Fig. 47. Each serum gave a comparable degree of haemadsorption on L cells. The cytolytic titre of the antisera is shown in Table 11. There was no significant difference in the titre of each serum.

Although most heterokaryons formed by fusing normal HeLa cells with UV-irradiated Ehrlich cells remained positive for Ehrlich antigen, some heterokaryons did become negative when gamma-irradiated HeLa cells were fused with UV-irradiated Ehrlich cells. The results of such an experiment are shown in Tables 12 and 13. Heavy UV-irradiation caused a decrease in the number of adherent heterokaryons although the degree of fusion was not affected as shown by the relatively constant numbers of single HeLa cells and HeLa homokaryons. Equal numbers of cells were used for each fusion. Because of heterokaryon death the degree of haemadsorption could not be counted for more than 3 days after fusion.

(vii) Actinomycin treatment of Ehrlich cells before fusion

A mouse bearing an Ehrlich ascites tumour was injected intraperitoneally with 100 ug of actinomycin. The cells were removed after 30 minutes and 10^7 were washed two times in 20 ml. of PBS. They were then fused with 5×10^6 normal HeLa cells using 8,000 HAU of virus. In spite of washing, there was enough residual actinomycin to cause the death of single HeLa cells and heterokaryons. Table 14 shows that single HeLa cells in fact died more rapidly than heterokaryons. All heterokaryons remained labelled for both HeLa and Ehrlich surface antigens (Fig. 48).

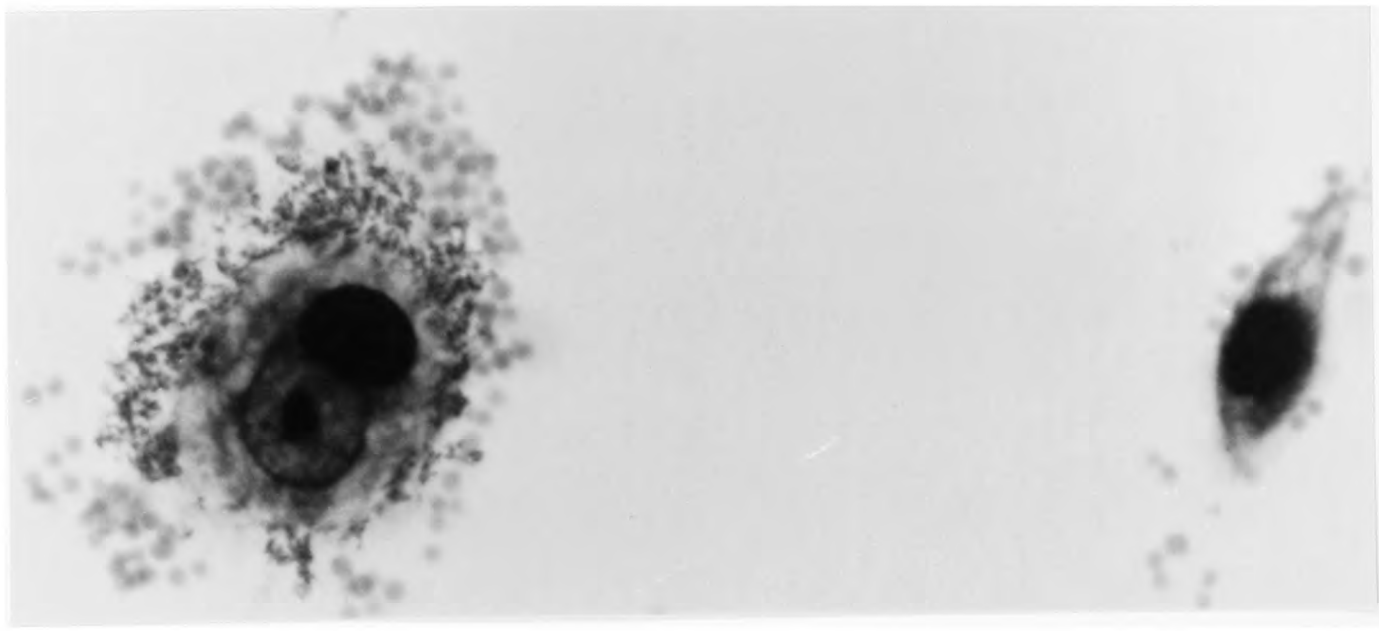

Figure 49. HeLa-L cell heterokaryon 24 hours after fusion. Both antigens are present as shown by the erythrocytes (L antigens) and bacteria (HeLa antigens). A single L cell is also present. x 720.

Table 15

Disappearance of Heterokaryons after Fusion of HeLa and L Cells

Cells labelled for	Days after fusion		
	1	2	3
L antigen only	7.4×10^3	1.8×10^4	1.9×10^4
Mononucleate	6.5×10^3	1.6×10^4	
Multinucleate	9.0×10^2	1.6×10^3	
HeLa antigen only	5.3×10^3	2.7×10^4	2.2×10^4
Mononucleate	4.7×10^3	2.6×10^4	
Multinucleate	6.0×10^2	6.3×10^2	
Both antigens	2.7×10^3	9.1×10^2	1.0×10^2
Mononucleate	6.0×10^2	2.8×10^2	
Multinucleate	2.1×10^3	6.3×10^2	
Total cells	1.5×10^4	4.6×10^4	4.1×10^4

4. HeLa-L cell Heterokaryons

(i) Mixed haemadsorption

Antigenic labelling of these heterokaryons was done by mixed haemadsorption in the same way as for HeLa-Ehrlich heterokaryons. Initially single labelling was used and suggested that no significant hybrid growth was occurring. 2×10^6 HeLa cells were fused with 4×10^6 L cells with 4,000 HAU of virus and the cells labelled daily with specific anti-L and anti-HeLa serum. The percentage of cells labelled with each antiserum was determined and from these figures the approximate percentage of cells labelled for both antigens could be calculated. 10% of mononuclear cells were labelled for both antigens one day after fusion, 6% 2 days after fusion and none 3 days after fusion. Counts of multinucleate cells showed that about 61% were labelled for both antigens 1 day after fusion, 27% 2 days after fusion, and 17% 4 days after fusion.

(ii) Double labelling

This technique showed the presence of each antigen on heterokaryons (Fig. 49) but also suggested that no hybrid growth was occurring after 3×10^6 HeLa and 3×10^6 L cells were fused with 4,000 HAU of virus (Table 15). Cloning experiments gave the same result. 5×10^6 HeLa and 5×10^6 L cells were fused with 8,000 HAU of virus. Double labelling on coverslips 24 hours after fusion showed that there were an average of 2.1×10^3 heterokaryons out of a total of 8.9×10^3 cells on the coverslips of which 6.1×10^2 were HeLa-L dikaryons and 8.0×10^2 heterosynkaryons. Cells were removed from 2 coverslips with 0.1%

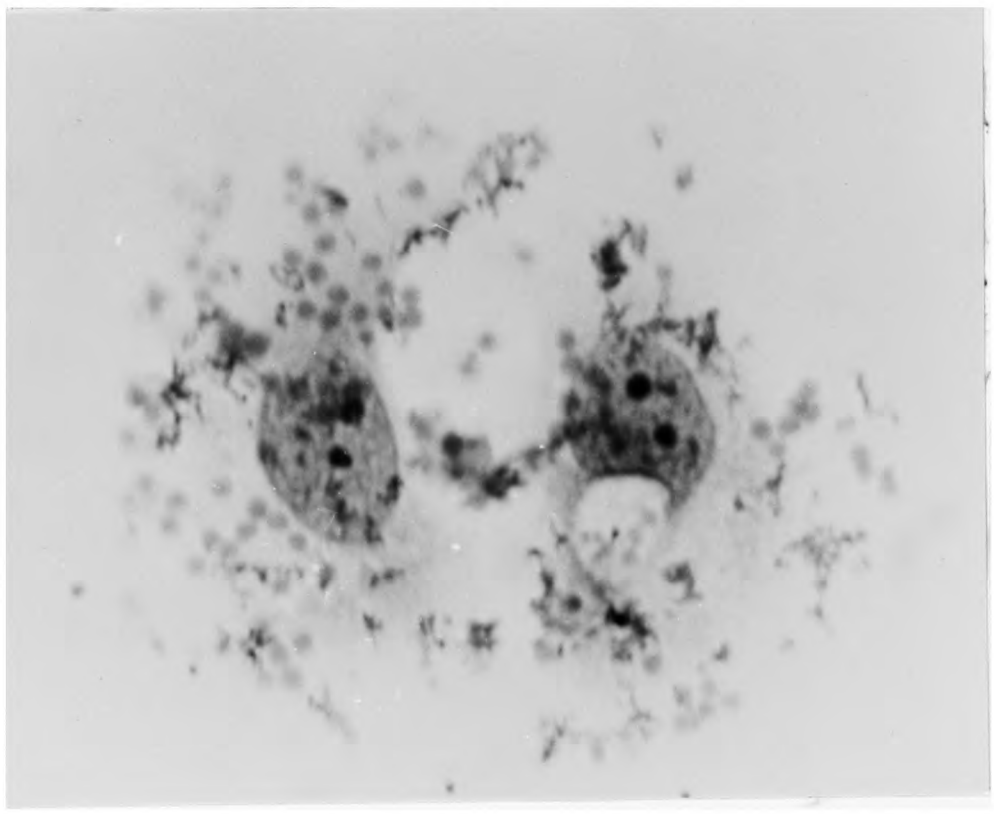

Figure 50. Pair of HeLa-L cell heterosynkaryons 3 days after cell fusion. They have been doubly labelled with erythrocytes and bacteria for HeLa and L antigens. x 720.

trypsin and the cells from each coverslip mixed with 5 x 10^5 HeLa cells previously given 8,000 r, and grown on coverslips in a Petri dish. Two days later there were some double labelled mononuclear cells but these were rare. One pair of mononuclear cells labelled for each antigen was seen (Fig. 50) but these were abnormal in appearance. Seven days later HeLa and L cells were growing well but there were very few doubly labelled cells and no clones of hybrid cells.

(iii) Use of mixed haemadsorption for cloning

In one experiment 5 x 10^6 L cells were fused with 10^4 HeLa cells using 4,000 HAU of virus. HeLa cells formed 0.2% of the total. It was hoped that all HeLa cells would be incorporated into heterokaryons and that clones labelled for HeLa antigen would represent hybrid cells. Fused cells were divided among 10 Petri dishes containing culture medium. Seven days later mixed haemadsorption was carried out on one Petri dish with anti-HeLa serum and some small labelled clones were present. A sterile glass rod tipped with rubber was used to scrape unlabelled cells off the Petri dish as well as possible. The remaining cells were removed with EDTA and seeded into another Petri dish. One week later many larger haemadsorption positive clones bearing HeLa antigens were present. The scraping was repeated and after another week of growth several coverslips from the Petri dish were checked for HeLa and L antigens. There were no remaining hybrid cells in a population containing 50% HeLa cells and 50% L cells. This also suggests that HeLa-L hybrid cells do not multiply and shows that mixed haemadsorption can be used to clone cells.

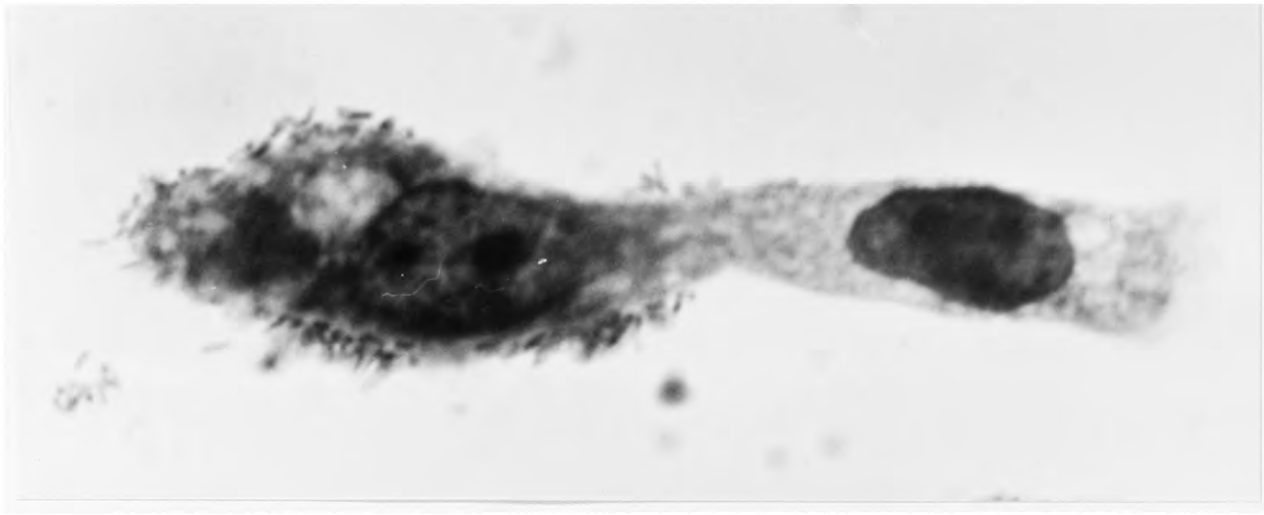

Figure 51. Fusion of a HeLa cell and an L cell on a monolayer. The HeLa cell is labelled with bacteria. The cells are in the process of fusing but mixing of antigens has not yet taken place. x 1300.

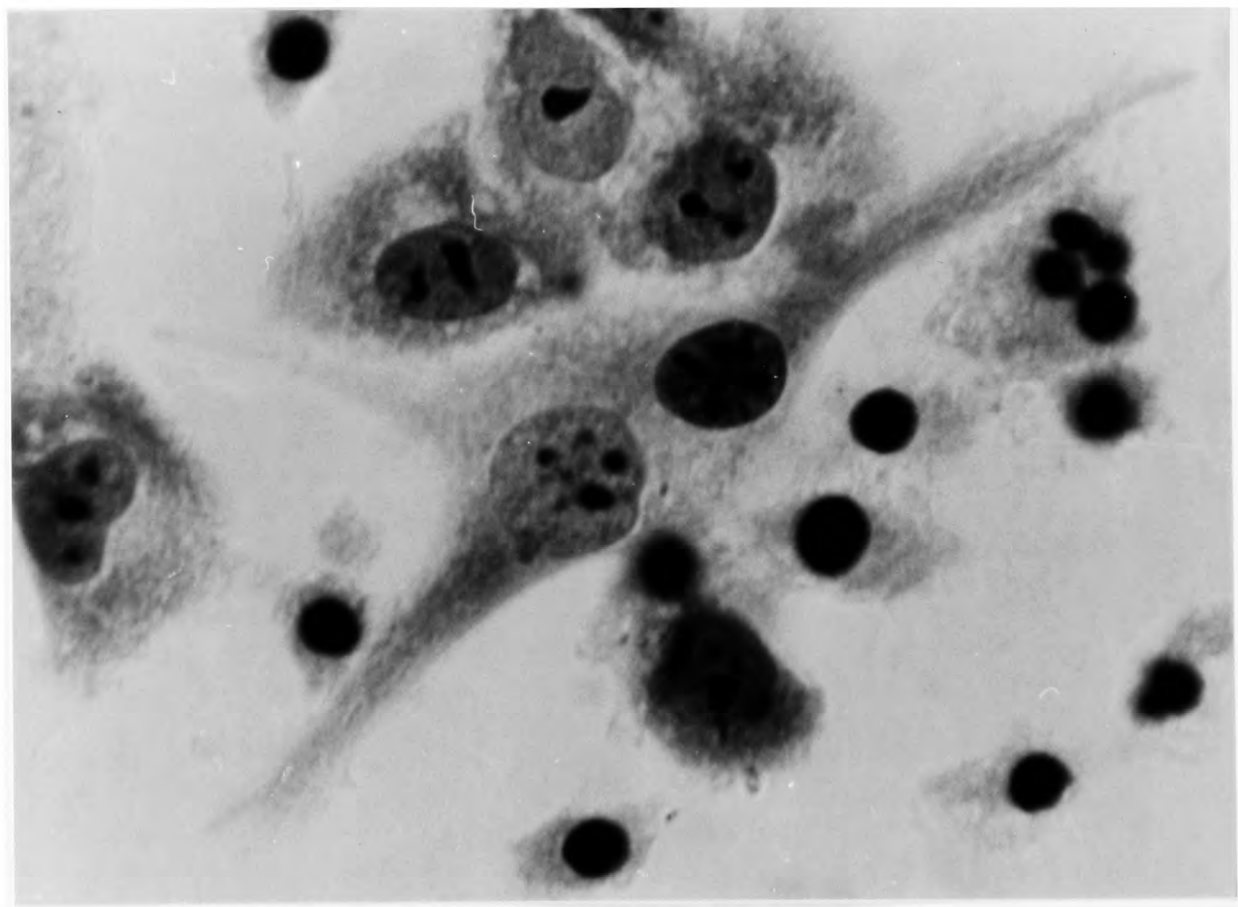

Figure 52. HeLa-L cell heterokaryon (centre) and single HeLa cells unlysed by 1/20 rabbit anti-L serum and guinea pig complement. Single L cells and an L cell homokaryon in the same field have been lysed. x 720.

(iv) Fusion on a monolayer

HeLa and L cells can be fused on a monolayer when each cell type is grown together. Moreover, one of the cell types can be labelled with specific antiserum and doubly sensitised bacteria and fusion will still take place as shown in Fig. 51. In this experiment 2 drops of virus suspension (0.05 ml.) with a concentration of 4,000 HAU per ml., were placed on the coverslip for 15 minutes at room temperature and the coverslip was returned to culture medium for one hour at 37°C.

(v) Cytolysis

Cytolysis of these heterokaryons was not carried out in detail because there is difficulty in regularly distinguishing HeLa and L nuclei. In some experiments heterokaryons could be distinguished and proved to be more resistant to anti-L serum and complement than single L cells or L homokaryons (Fig. 52).

5. Ehrlich-L Heterokaryons

Studies on these heterokaryons were hampered by the difficulty in obtaining specific antiserum for each cell type. An attempt was made to absorb rabbit anti-Ehrlich serum with L cells but this was not successful in removing all antibody for L cells. Unabsorbed anti-Ehrlich serum at a dilution of 10^{-5} resulted in the haemadsorption of 24 erythrocytes to each L cell on a monolayer. The same dilution of antiserum absorbed with 10^{7} L cells per ml. still resulted in the adherence of 6.6 erythrocytes to each L cell. In vivo absorption of antiserum was suggested by the experiments of Gorer, Tuffrey, and

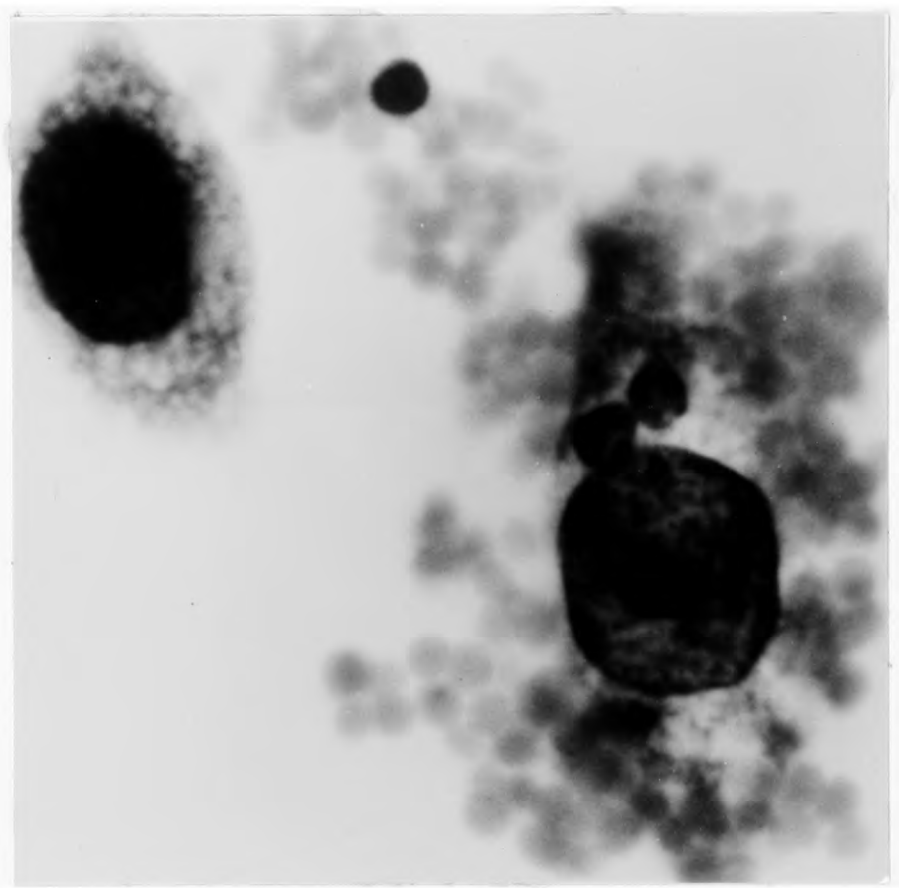

Figure 53. HeLa-hen erythrocyte heterokaryon labelled for hen antigens by mixed haemadsorption 24 hours after cell fusion. A single HeLa cell is unlabelled. x 2,000.

Batchelor (1962). They used this method to demonstrate "x antigens" on mouse leukaemia cells. A mouse was therefore injected intravenously with 0.1 ml. of rabbit anti-L serum and the animal bled out one hour later. The mouse serum was titrated for persistent rabbit antibody by the usual mixed haemadsorption method. There was no haemadsorption on mouse embryo fibroblasts or Ehrlich cells from non-inbred mice but good labelling was still obtained on L cell monolayers with the mouse serum diluted 1/10 or 1/100. This antigen was also present on the surface of heterokaryons formed by fusing 2×10^6 L cells with 10^7 Ehrlich cells with 4,000 HAU of virus. Because no specific anti-Ehrlich serum was available mononuclear hybrid cells could not be detected.

6. HeLa-erythrocyte Heterokaryons

(i) Hen erythrocytes

In initial experiments 3×10^6 HeLa cells were fused with 2×10^7 hen erythrocytes using 1,000 HAU of virus. The virus dose was later decreased to 250 or 500 HAU with better results. Unabsorbed rabbit antiserum to hen erythrocytes gave excellent labelling without cross-reaction on HeLa cells, at a concentration of 10^{-3}. Fig. 53 shows mixed haemadsorption for hen antigen on a HeLa-hen erythrocyte heterokaryon one day after fusion. There are two hen nuclei in the heterokaryon and these have not yet begun to expand. The cell is labelled over its whole surface for hen antigen while an adjacent single HeLa cell is unlabelled. The small labelled particle on the coverslip may

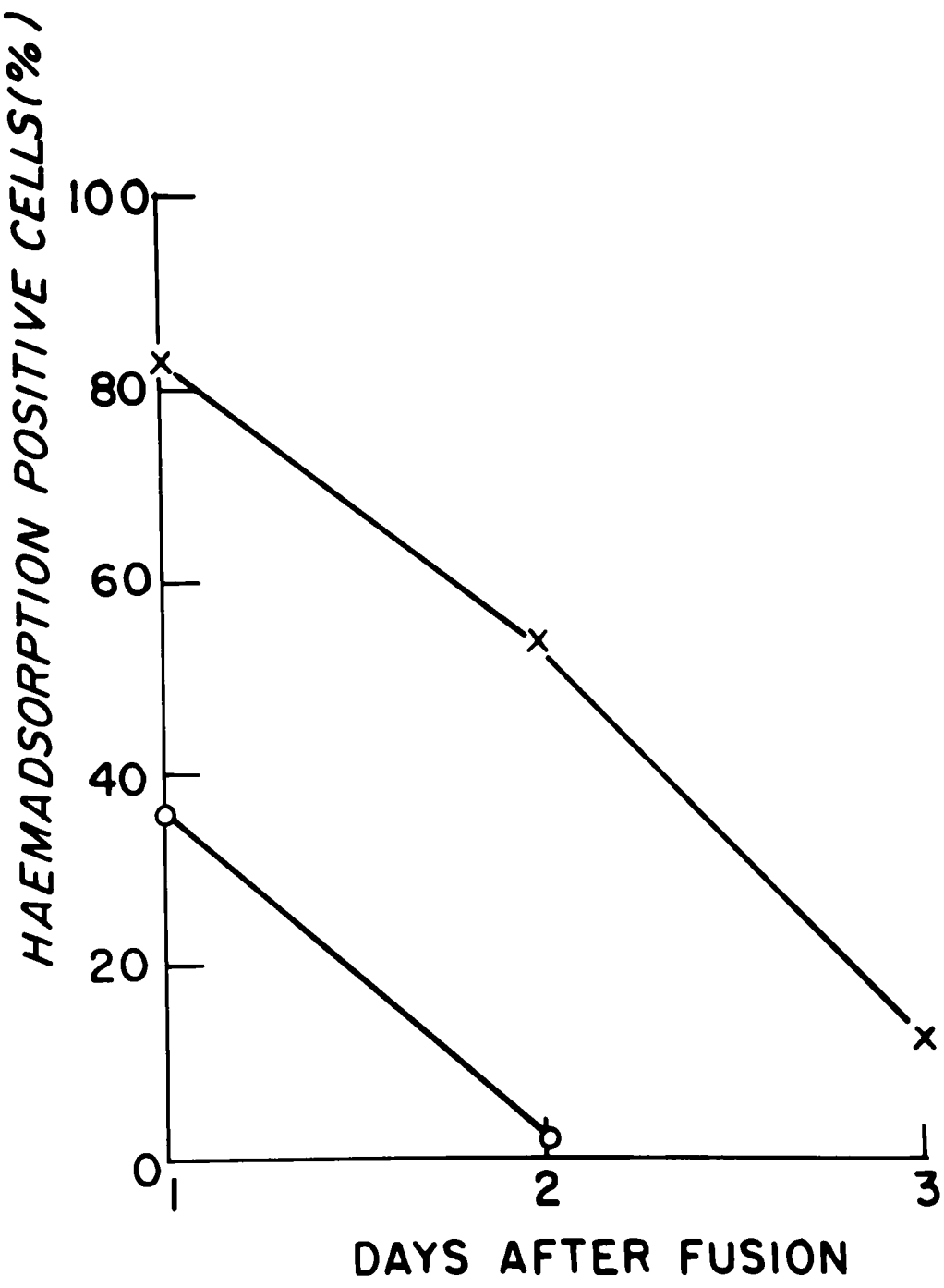

Figure 54. Percentage of HeLa-hen erythrocyte dikaryons (upper line) and single HeLa cells (lower line) labelled for hen antigens by mixed haemadsorption.

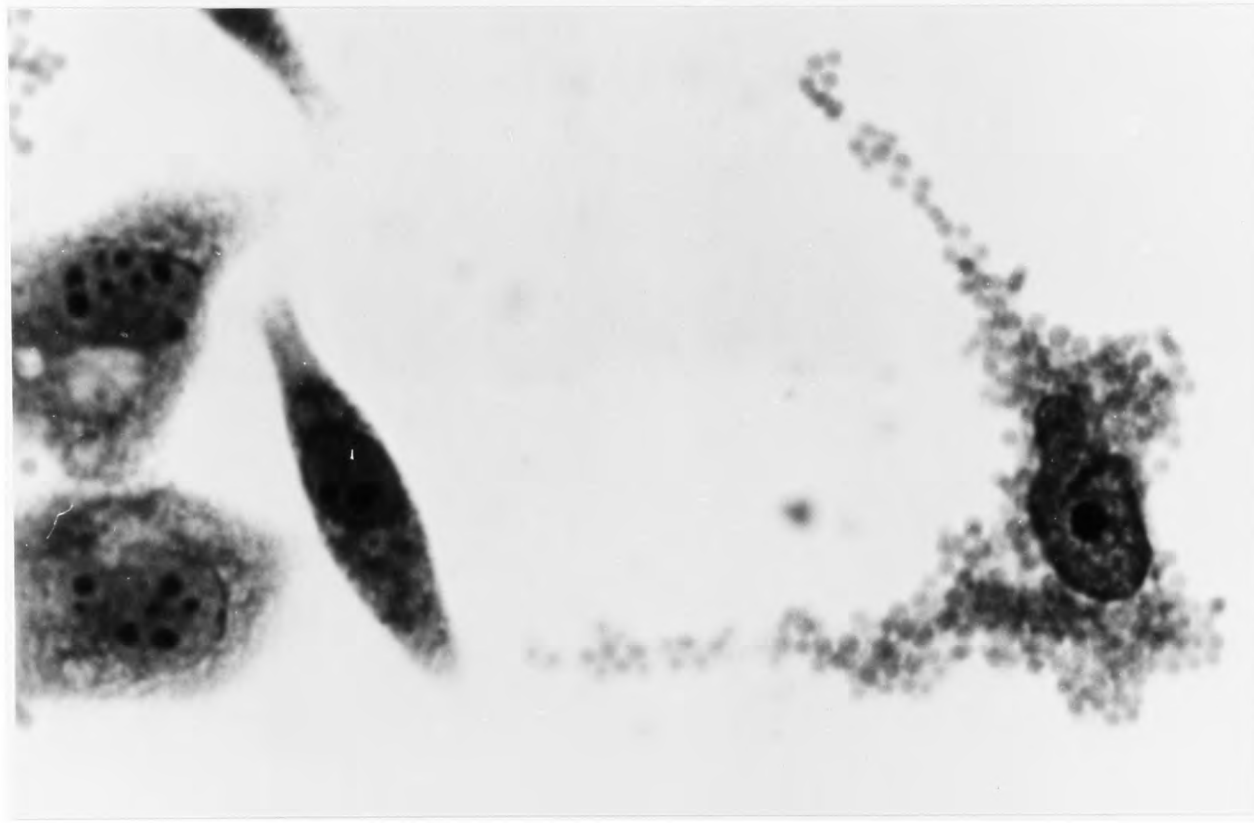

Figure 55. Mixed haemadsorption for hen antigens on a HeLa-hen erythrocyte heterokaryon 2 days after fusion. The small projection from the upper margin of the HeLa nucleus may represent the hen erythrocyte nucleus. x 720.

represent a hen nucleus in HeLa cytoplasm as described by Harris (1967).

When heterokaryons were labelled daily for hen antigen it became apparent that this antigen was disappearing as shown in Fig. 54. A cell was counted as positive if it had more than 10 adherent erythrocytes. One day after fusion 83% of dikaryons (1 HeLa and 1 chick nucleus) were labelled for hen antigen and 96% of all heterokaryons. This decreased to 54% of dikaryons and 68% of heterokaryons 2 days after fusion. It was of interest that many HeLa cells which did not contain hen nuclei were nevertheless labelled for hen antigen. The following reasons for labelling on single HeLa cells were considered:

1. Chick antigen might be left on the surface of cells by the virus since it was grown in the allantoic cavity of fertile hens' eggs. This was tested by fusing HeLa cells with each other. Haemadsorption with anti-erythrocyte serum 24 hours after fusion was completely negative and so the virus did not carry chick antigen.

2. A hen erythrocyte nucleus might be present but hidden under the much larger HeLa nucleus. This seemed most unlikely since all heterokaryons were well flattened and a hen nucleus would be forced to a peripheral position by the HeLa nucleus.

3. The hen nucleus might have fused with the HeLa nucleus with persistence of hen antigen. Nuclear fusion can certainly occur in these heterokaryons (Harris et al., 1966; Harris, 1967) and some examples were seen in these experiments in which nuclear fusion seemed to have taken place (Fig. 55). However, this was usually seen 48 or more hours after fusion and appeared to be a rare event.

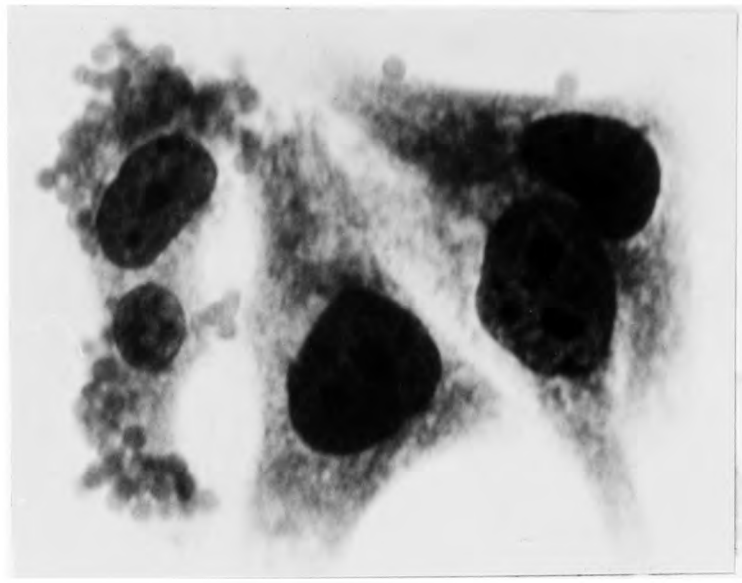

Figure 56. Mixed haemadsorption for hen antigens on HeLa-hen erythrocyte heterokaryons 48 hours after cell fusion. Labelling is confined to the heterokaryon in spite of contact with single HeLa cells. x 720.

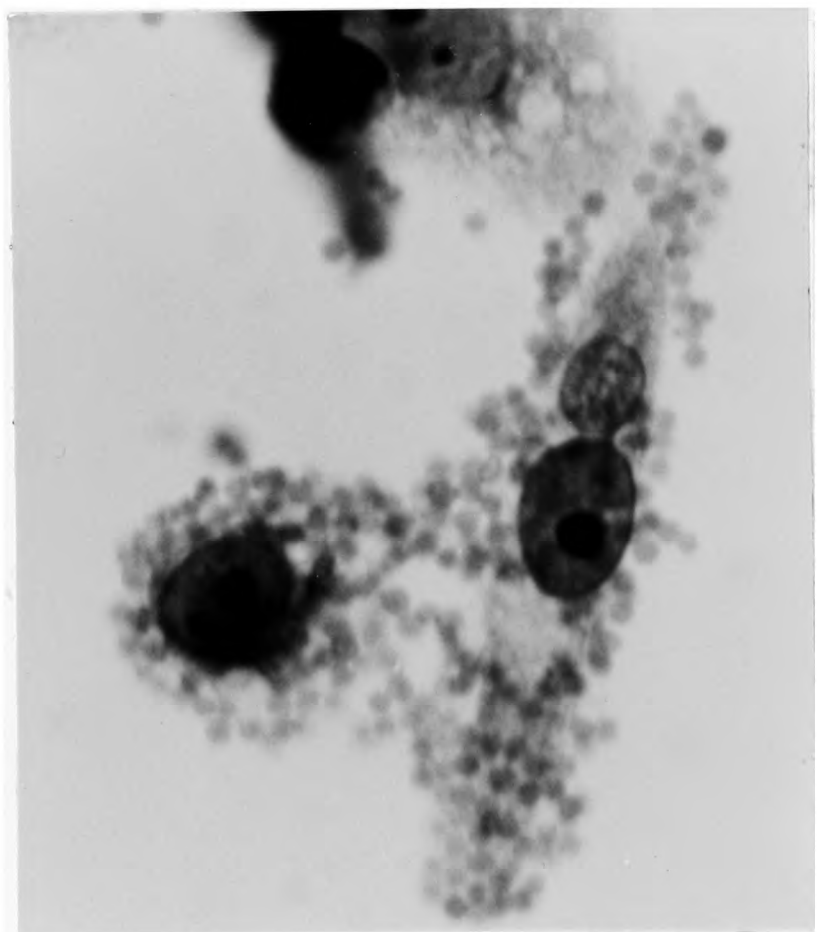

Figure 57. Division of a HeLa-hen erythrocyte heterokaryon 48 hours after fusion. The heterokaryon and the single HeLa cell are labelled for hen antigens by mixed haemadsorption. x 720.

4. Passage of hen antigen from a heterokaryon to neighbouring cells on a monolayer might occur. This seemed unlikely since many examples were seen in which a heterokaryon strongly labelled for hen antigen was actually in contact with unlabelled single HeLa cells (Fig. 56).

5. Division of a heterokaryon to give a single HeLa cell and a smaller heterokaryon, both bearing hen antigen, might occur. Such division has been observed (Harris, 1967) and hen antigen can be detected on single HeLa cells soon after apparent separation from heterokaryons (Fig. 57), but this is a rare event and is usually seen 48 or more hours after fusion.

6. Hen antigen might be left on the cell surface as a result of partial membrane fusion without a hen nucleus actually entering the HeLa cell. This seemed to be the most likely explanation for most of the labelling on single HeLa cells and will be considered further in the Discussion.

The proportion of single HeLa cells labelled for hen antigens was variable but often considerable 24 hours after fusion. However, this antigen had disappeared 48 hours after fusion as shown in Fig. 54.

Hen cells carry Forssman antigen. This was shown by the fact that rabbit anti-hen erythrocyte serum used in these experiments, which lysed hen erythrocytes to a dilution of 1/1,000, also lysed erythrocytes from the Forssman-positive sheep to a dilution of 1/256. Exhaustive absorption of the anti-hen erythrocyte serum with washed, packed sheep erythrocytes, removed all haemolytic antibody for the sheep erythrocytes

Table 16

Fusion of Chick Embryo Erythrocytes with Normal and Gamma-Irradiated HeLa Cells

Days after fusion	Number of dikaryons per coverslip	
	No radiation to HeLa cells	8,000 r to HeLa cells
1	4.0×10^2	1.2×10^3
2	1.3×10^2	9.5×10^2
3	75	4.5×10^2

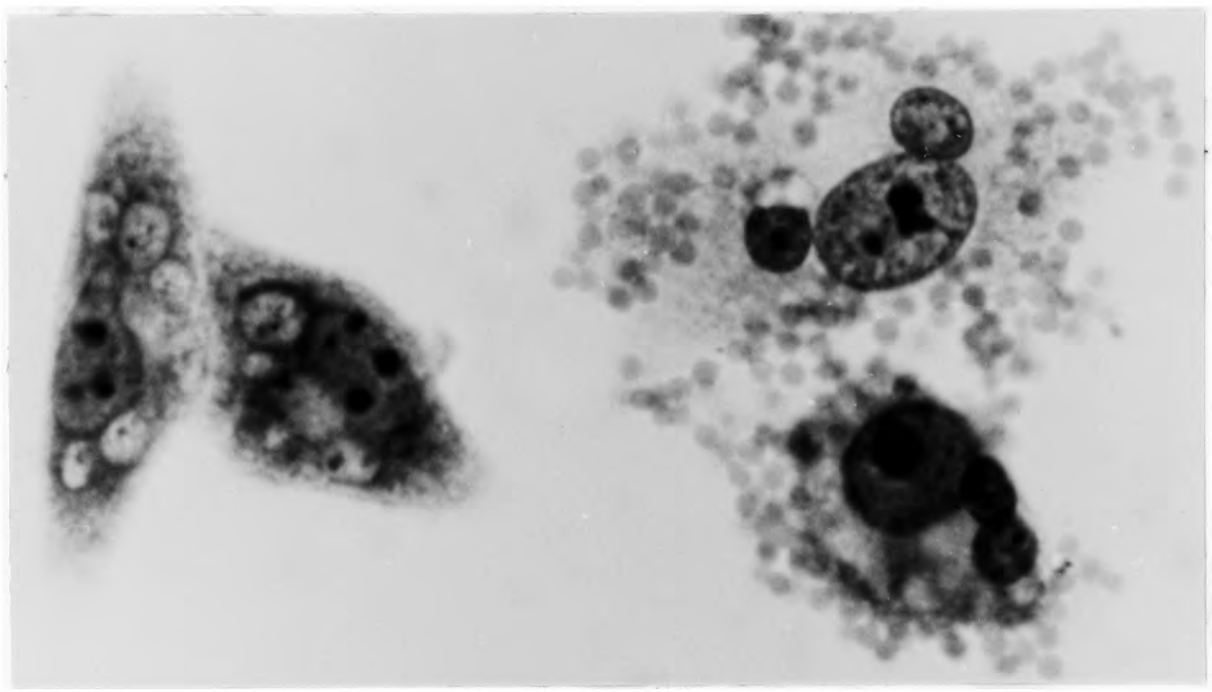

Figure 58. Mixed haemadsorption for chick antigens 2 days after the fusion of chick embryo erythrocytes and gamma-irradiated HeLa cells. There are 2 heterokaryons labelled for chick antigens and 2 unlabelled HeLa cells showing some micronucleation. x 600.

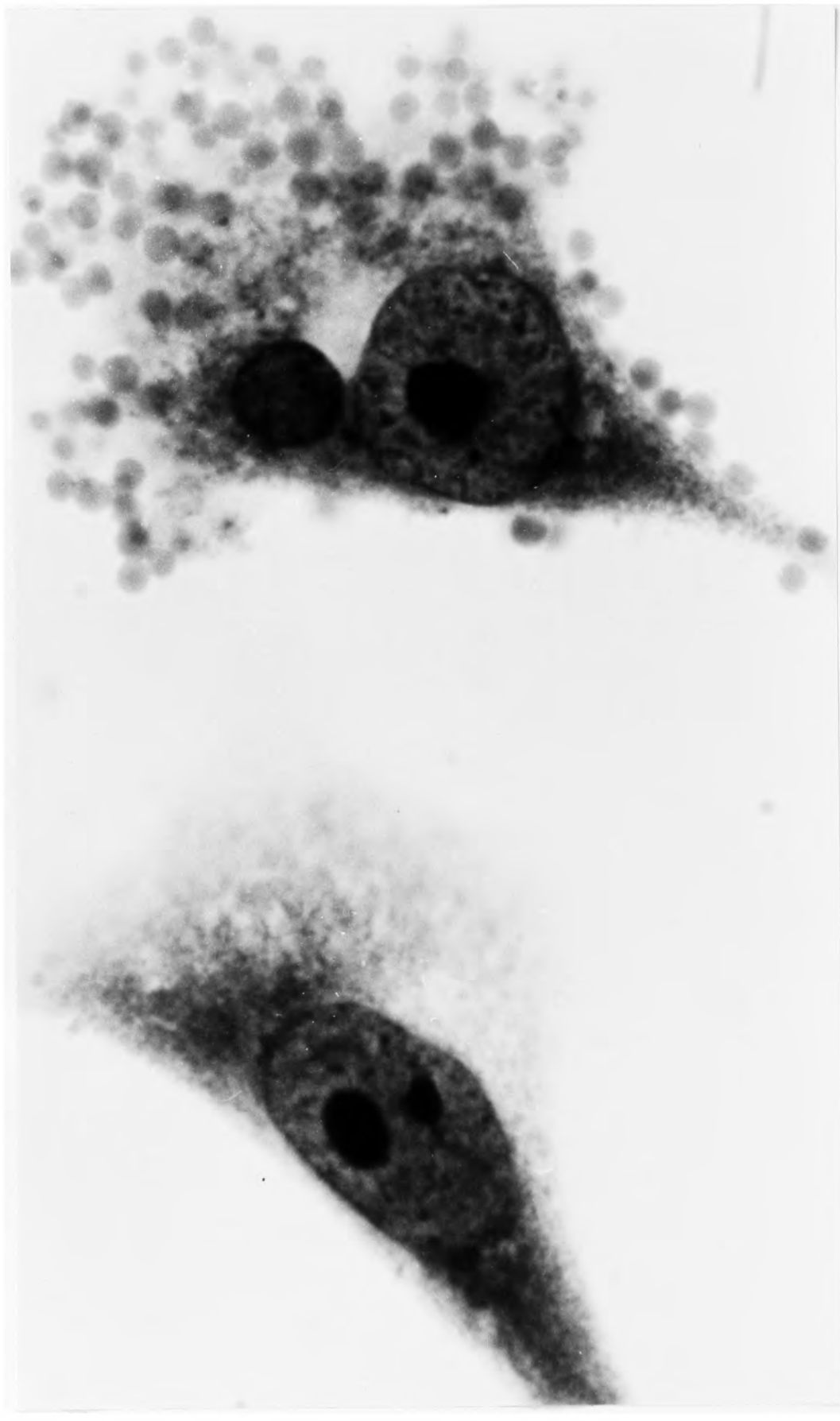

Figure 59. Mixed haemadsorption for chick antigen on a HeLa-chick erythrocyte heterokaryon 2 days after fusion. An unlabelled single HeLa cell is present in the same field. x 2,000.

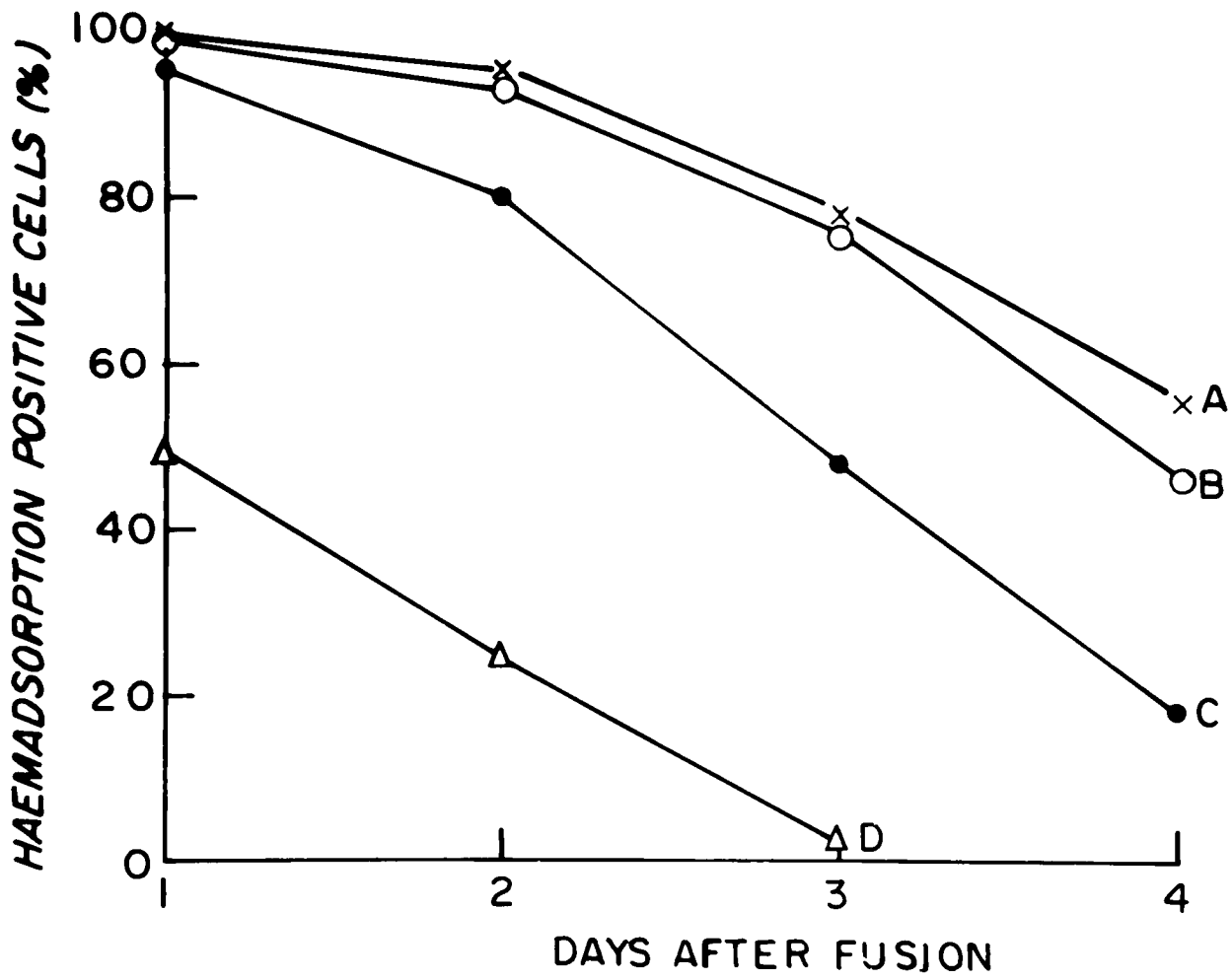

Figure 60. Mixed haemadsorption for chick antigen on HeLa-chick erythrocyte heterokaryons. Only dikaryons with one HeLa and one chick nucleus have been counted.
A. Normal HeLa cells fused with normal erythrocytes.
B. Gamma-irradiated HeLa cells (4,000 r) fused with normal erythrocytes.
C. Gamma-irradiated HeLa cells fused with UV-irradiated erythrocytes (10,000 ergs/mm^2).
D. Single HeLa cells in experiment in which gamma-irradiated HeLa cells were fused with normal erythrocytes.

but had no effect on the degree of mixed haemadsorption on HeLa-hen erythrocyte heterokaryons. Thus it appeared that species antigens were being detected on heterokaryons and were disappearing.

(ii) Chick embryo erythrocytes

For further experiments HeLa cells were fused with chick embryo erythrocytes since these have been shown by Harris (unpublished observations) to fuse much more readily than adult erythrocytes. Usually 5×10^6 HeLa cells were fused with 5×10^7 embryo erythrocytes using 250 or 500 HAU of virus. It was found that heterokaryons were more plentiful and lived longer if HeLa cells were gamma-irradiated before fusion. The results of such an experiment are shown in Table 16.

The same numbers of cells were used for each fusion. The persistence of more heterokaryons several days after fusion made it easier to count many cells in haemadsorption experiments. Some irradiated HeLa cells developed micro-nuclei several days after irradiation and these had to be differentiated from enlarging chick nuclei (Fig. 58) but in most cases this was not difficult.

For initial experiments on these heterokaryons antiserum to hen erythrocytes was used for mixed haemadsorption. In later experiments rabbit antiserum to chick embryo erythrocytes was employed and gave the same results. As with HeLa-hen erythrocyte heterokaryons, labelling for chick antigens often occurred over the whole cell surface (Fig. 59), and disappeared from dikaryons and single HeLa cells in the same way (Fig. 60). Chick antigen disappeared slightly more slowly from single gamma-irradiated HeLa cells (Fig. 60) than from normal HeLa cells (Fig.

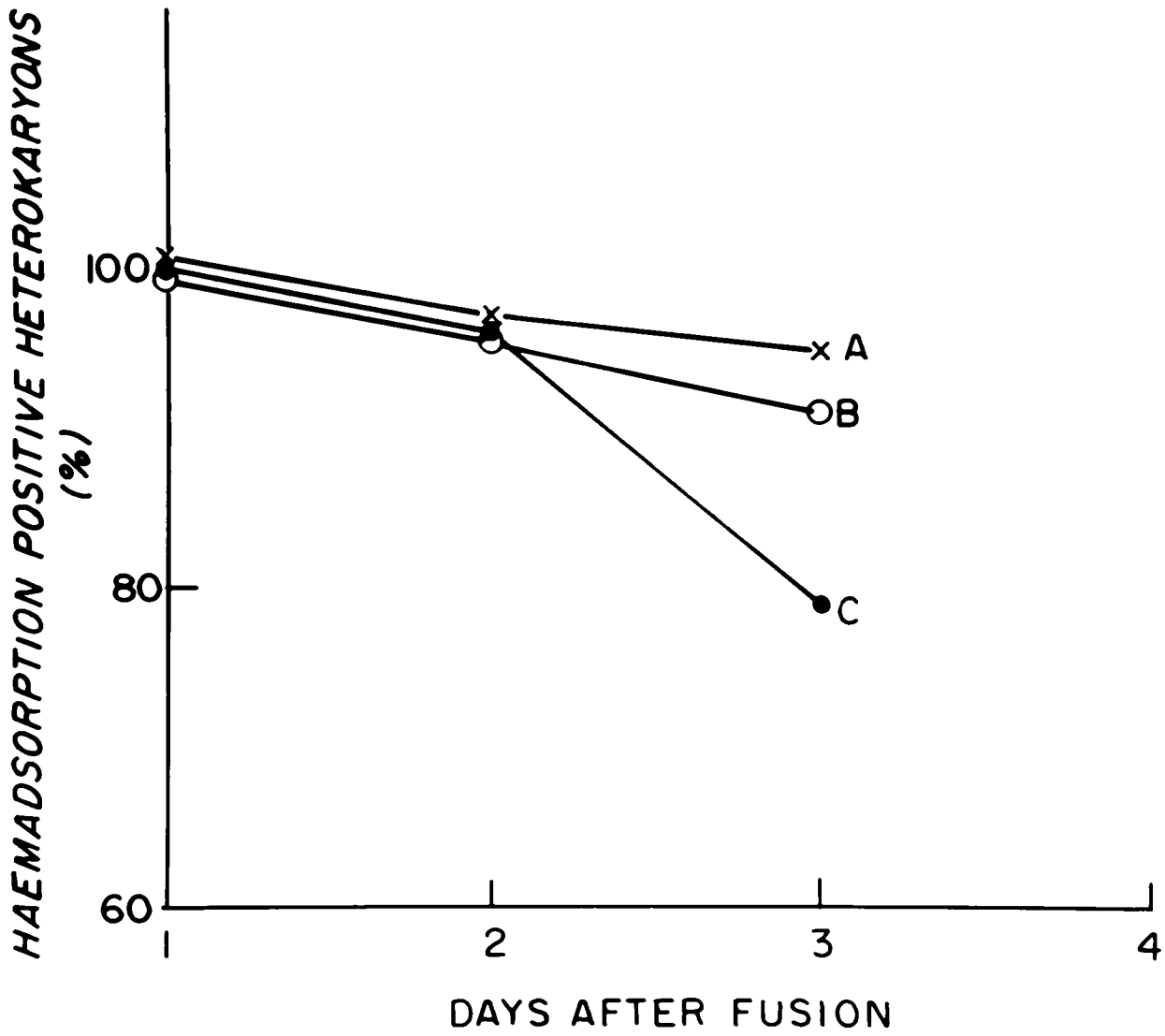

Figure 61. Mixed haemadsorption for chick antigen on heterokaryons formed by fusing chick embryo erythrocytes with gamma-irradiated HeLa cells. The heterokaryons contained 4 chick nuclei (A), 3 chick nuclei (B) and 2 chick nuclei (C).

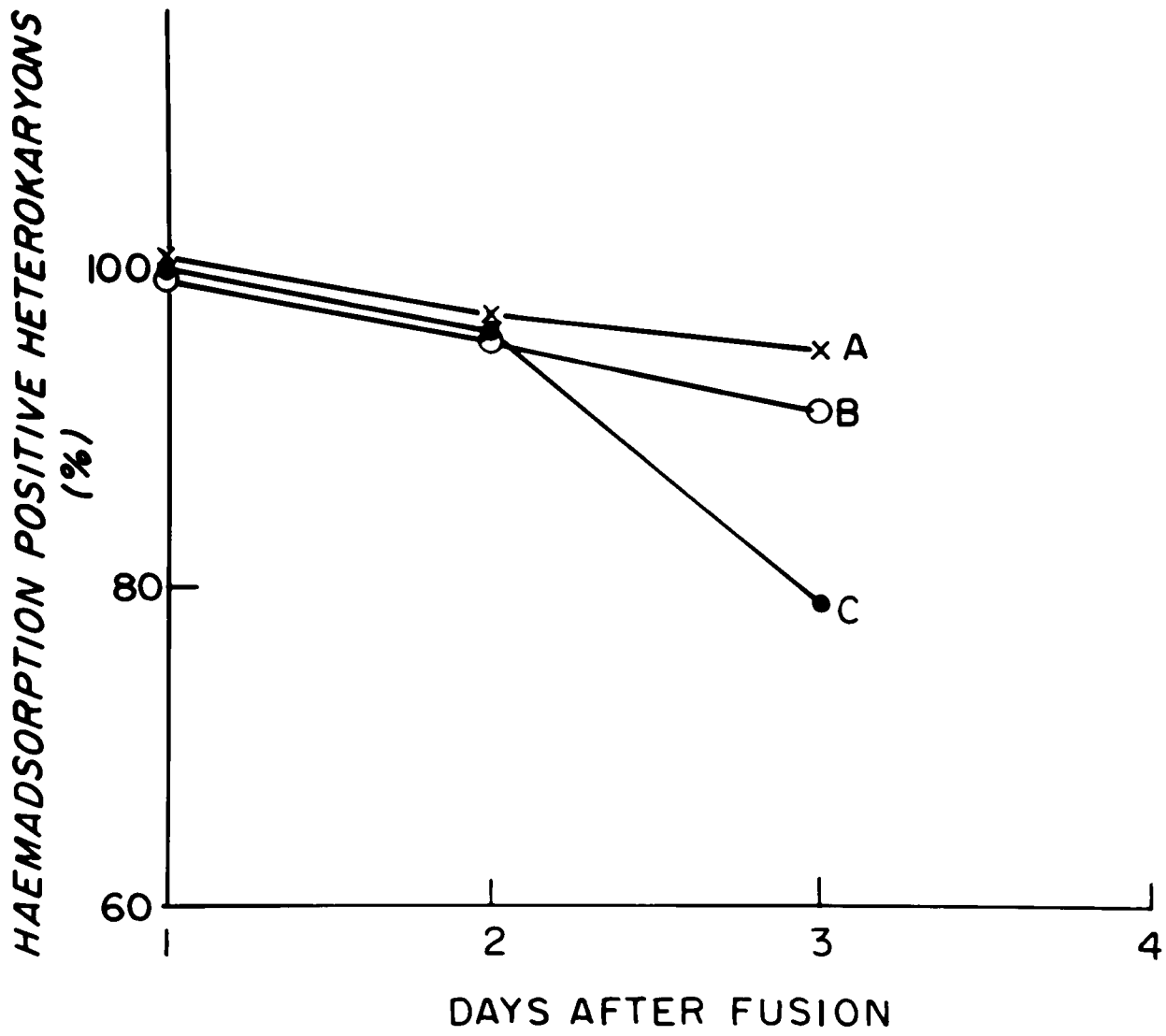

Figure 61. Mixed haemadsorption for chick antigen on heterokaryons formed by fusing chick embryo erythrocytes with gamma-irradiated HeLa cells. The heterokaryons contained 4 chick nuclei (A), 3 chick nuclei (B) and 2 chick nuclei (C).

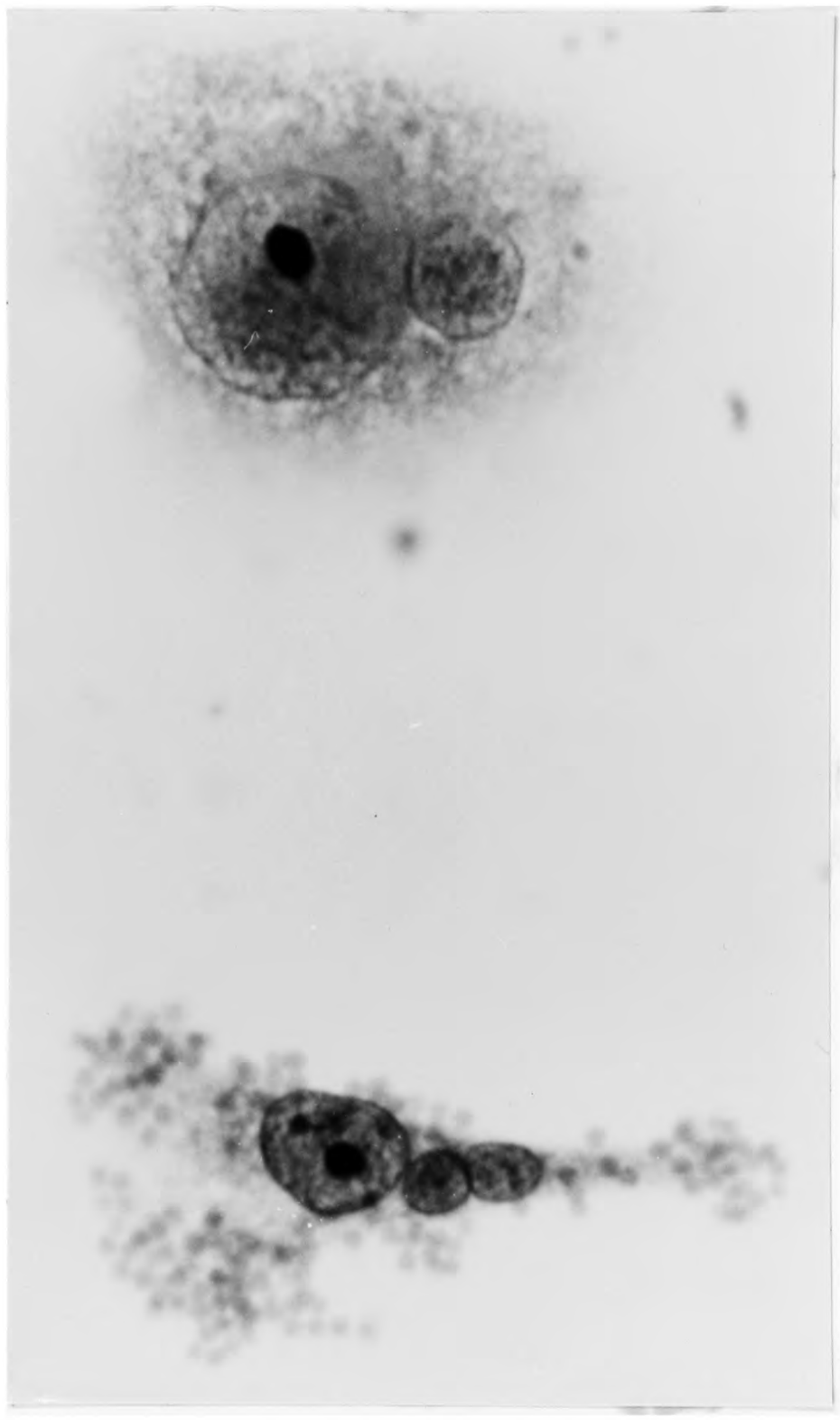

Figure 62. HeLa-chick erythrocyte heterokaryons 48 hours after cell fusion. One is strongly labelled for chick antigens by mixed haemadsorption while the other, containing the more expanded chick nucleus, is unlabelled. x 720.

54). The rate of antigen disappearance was also related to the number of chick nuclei in the heterokaryon (Fig. 61). Often adjacent dikaryons showed a marked difference in the amount of chick antigen on the cell surface (Fig. 62). In this case chick antigen has been lost from the heterokaryon in which there is greater enlargement of the chick nucleus.

An attempt was made to lyse HeLa-chick erythrocyte heterokaryons with rabbit antiserum which lysed chick embryo erythrocytes to a dilution of 1/1,000. Coverslips bearing a population in which 52% of cells were heterokaryons were placed in 1/2 and 1/10 dilutions of antiserum with 1/20 guinea pig serum for 2 hours at 37°C. There was no visible cytolysis of heterokaryons. However, there was some complement fixation as shown by weak immune adherence of human group O erythrocytes to these heterokaryons.

(iii) Other erythrocytes

It was of interest to see whether foreign antigen could be detected on the surface of HeLa cells after virus was added to a mixture of HeLa cells and mammalian erythrocytes. In the first experiment 2×10^6 HeLa cells were mixed with 10^9 washed mouse erythrocytes and 4,000 HAU of virus added. The usual method for cell fusion was followed with an initial period at 4°C to allow cell agglutination to occur. Cells on coverslips were transferred to fresh medium after 12 hours. Mixed haemadsorption was carried out 24 hours after fusion with anti-Ehrlich serum as used for HeLa-Ehrlich heterokaryons. This antiserum lysed Ehrlich cells to a dilution of 1/1,000 and mouse erythrocytes to a

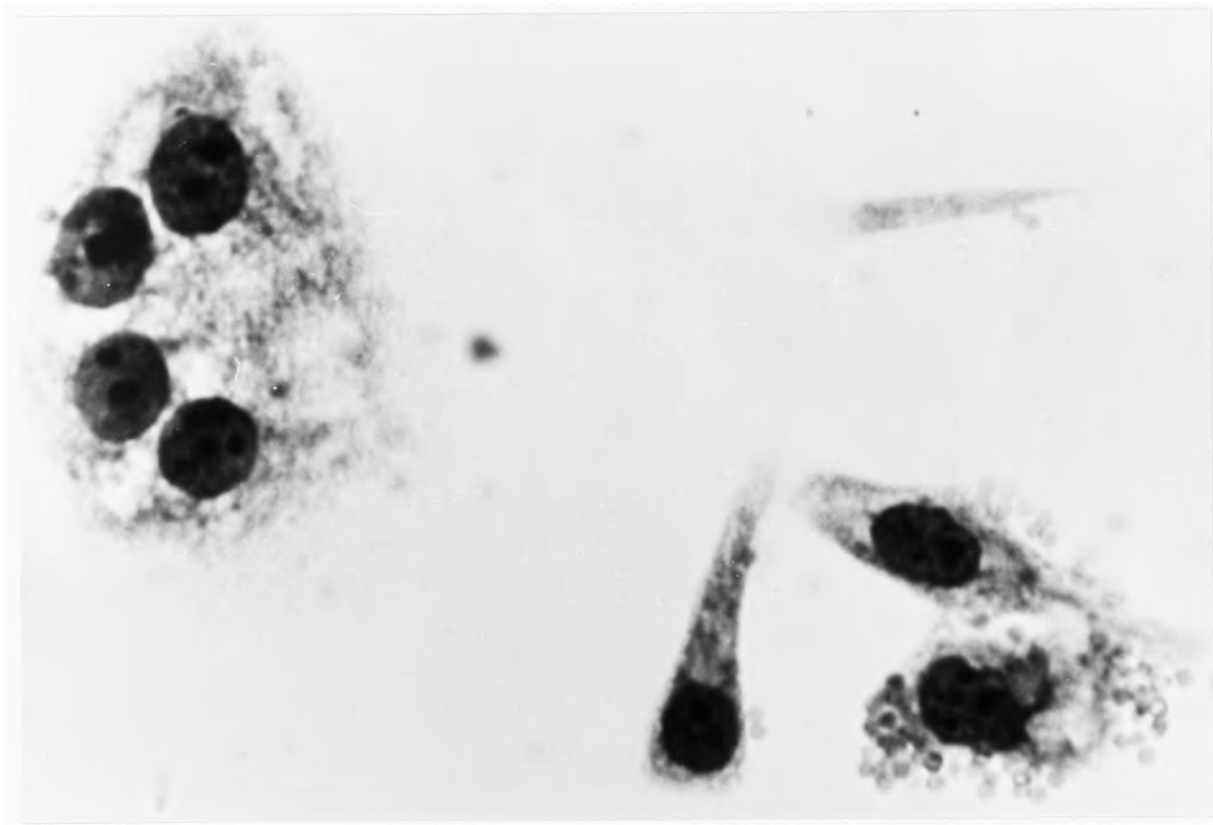

Figure 63. Mixed haemadsorption on HeLa cells with rabbit anti-sheep erythrocyte serum 24 hours after mixing HeLa cells, sheep erythrocytes and inactivated Sendai virus. A HeLa cell in the lower right corner is labelled for sheep antigen. x 600.

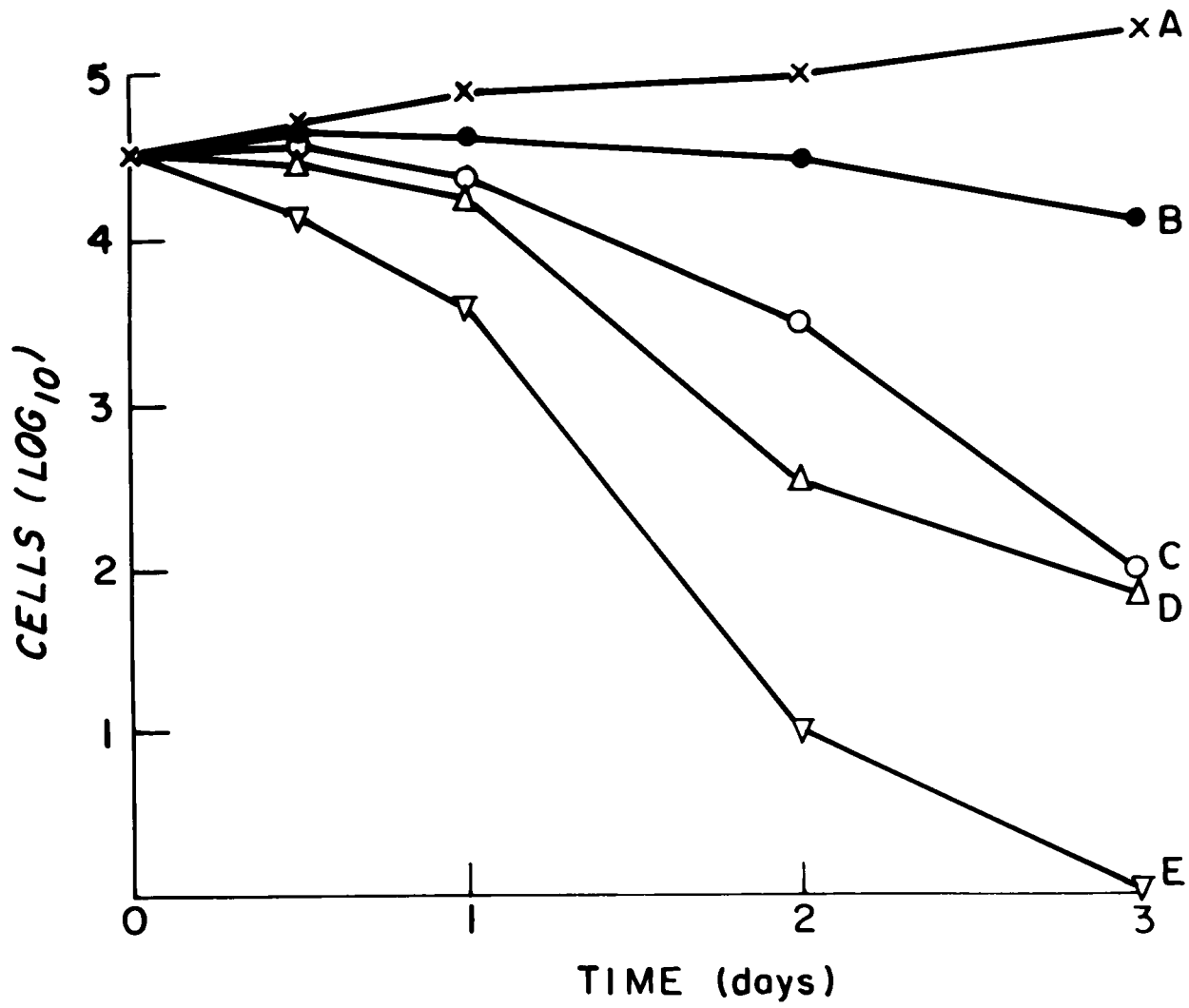

Figure 64. Number of surviving HeLa cells per coverslip after addition of actinomycin D to the culture medium.

A- control
B- 0.01 ug/ml
C- 0.025 ug/ml
D- 0.05 ug/ml
E- 0.10 ug/ml

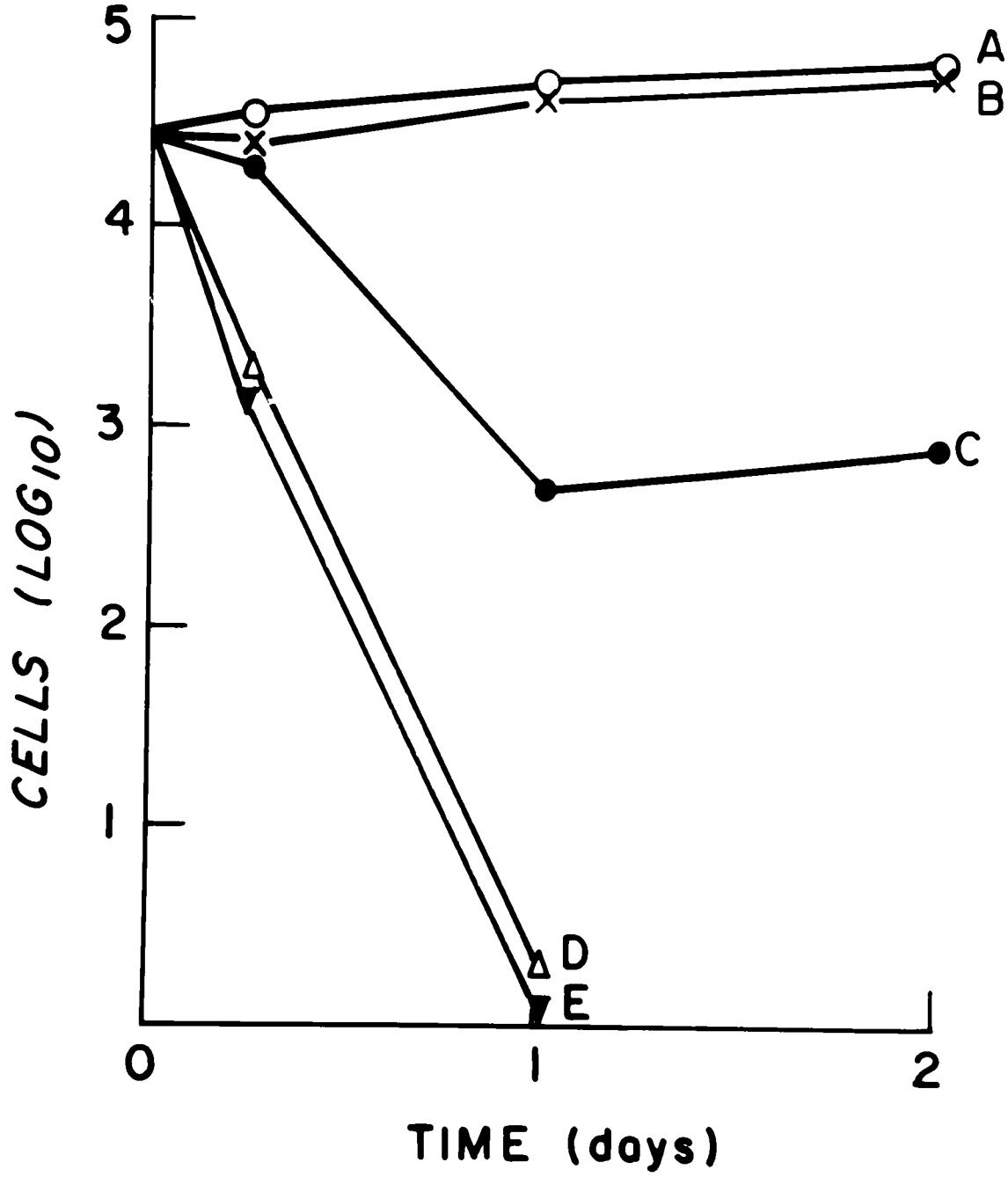

Figure 65. Number of surviving HeLa cells per coverslip after addition of puromycin to the culture medium.

A - control
B - 0.01 ug/ml
C - 0.10 ug/ml
D - 1.0 ug/ml
E - 10 ug/ml

dilution of 1/128. No mouse antigen was detectable on HeLa cells with this serum.

In the second experiment 5×10^6 HeLa cells were mixed with 2×10^8 sheep erythrocytes and 2,000 HAU of virus was added. There was a considerable amount of haemolysis as one would expect. Twenty-four hours after fusion coverslips were rinsed in PBS and mixed haemadsorption carried out with rabbit antiserum to sheep erythrocytes at a dilution of 10^{-3}. At this concentration the serum gave no mixed haemadsorption on single HeLa cells but still lysed sheep erythrocytes. 20% of HeLa cells in this fusion experiment showed some degree of haemadsorption for sheep antigen (Fig. 63) but there was no labelling after a further 24 hours in fresh culture medium.

(iv) Effect of actinomycin D and puromycin

An attempt was made to study the effect of puromycin and actinomycin D on the disappearance of chick antigen from HeLa-chick erythrocyte heterokaryons. For such an experiment it would be necessary to find a concentration which would allow cells to remain adherent to coverslips and yet have some effect on protein synthesis (puromycin) and RNA synthesis (actinomycin). The effect of different concentrations of these drugs on the sticking of HeLa cells is shown in Figs. 64 and 65. A dose of actinomycin as low as 0.01 µg./ml. produced definite morphological changes in HeLa nuclei after 9 hours of exposure, but caused no visible reduction in the degree of autoradiographic labelling for DNA, RNA, or protein synthesis in comparison to untreated controls after 24 hours of exposure. 0.1 µg./ml. caused quite rapid cell death

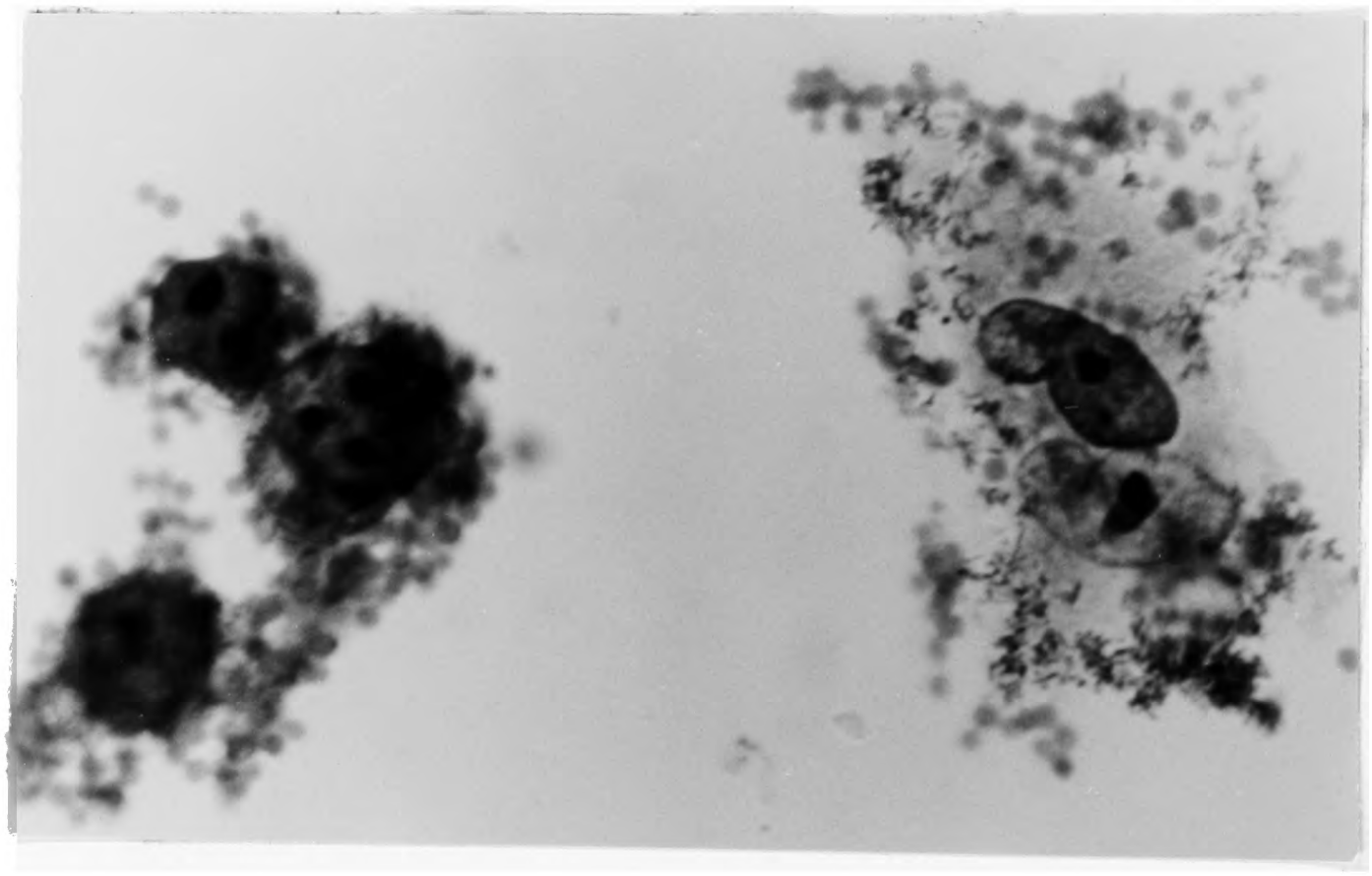

Figure 66. HeLa-chick fibroblast heterokaryon labelled for HeLa antigens with erythrocytes and for chick antigens with bacteria 24 hours after cell fusion. Some single HeLa cells are also present. x 720.

and so was too high a dose for experiments on heterokaryons.

Puromycin also caused cell death with a dose as low as 0.1 µg/ml. and some cells became vacuolated after only 6 hours of exposure to this concentration of the drug. One therefore questions the validity of results obtained with the much higher concentrations of puromycin used in most experiments for suppression of protein synthesis. 0.04 µgm of puromycin per ml., a dose which prevented multiplication without causing rapid cell death, had no apparent effect on autoradiographic labelling for DNA, RNA, or protein synthesis after 24 hours of exposure. It was therefore impossible to carry out meaningful experiments on the disappearance of chick antigen from HeLa-chick erythrocyte heterokaryons with these agents.

7. HeLa-Chick Fibroblast Heterokaryons

Studies on this system were hindered by the difficulty of obtaining good haemadsorption on monolayers of chick fibroblasts with rabbit anti-fibroblast serum. Some fibroblasts were always unlabelled although most had adherent erythrocytes. Furthermore, heterokaryons seemed to disappear from culture fairly rapidly and most were gone within 3 days of fusion. Usually about 4×10^6 HeLa cells were fused with 4×10^6 fibroblasts using 1,000 or 2,000 HAU of virus. Unabsorbed anti-fibroblast serum was specific for chick fibroblasts without cross-reaction on HeLa cells at a dilution of 10^{-3}. Both HeLa and chick antigens were present on these heterokaryons as shown by double labelling (Fig. 66) and some mononuclear cells carrying both antigens were also

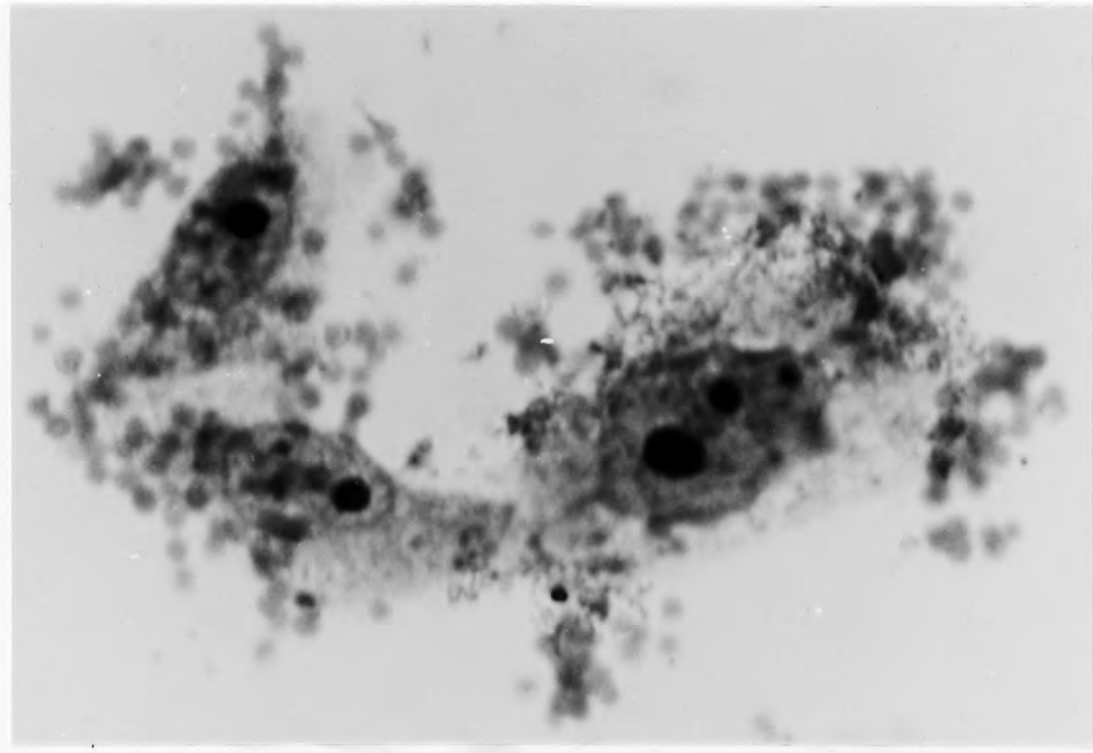

Figure 67. HeLa-chick fibroblast heterosynkaryon (right) labelled for HeLa antigens with erythrocytes and for chick antigens with bacteria 24 hours after cell fusion. x 720.

Table 17

Mixed Haemadsorption for Chick Antigen on HeLa-Chick Fibroblast Heterokaryons

Experiment number	Labelled dikaryons per coverslip (%)		
	1 day after fusion	2 days after fusion	3 days after fusion
1	94	93	A
2	92	B	89
3	100	100	100
4	88	83	A

A. Not enough remaining dikaryons to count

B. No count done

Table 18

Cytolysis of HeLa-chick Fibroblast Heterokaryons

Cells counted	Serum concentration	Number of cells per coverslip	
		Control (normal rabbit serum)	Anti-chick serum
HeLa	1/10	2.5×10^3	1.8×10^3
	1/20	2.4×10^3	3.4×10^3
	1/40	5.3×10^3	3.7×10^3
	1/80	2.5×10^3	4.2×10^3
Chick fibroblasts	1/10	3.0×10^3	0
	1/20	2.6×10^3	0
	1/40	5.7×10^3	0
	1/80	2.4×10^3	0
Hetero-karyons	1/10	4.6×10^2	66
	1/20	5.5×10^2	3.1×10^2
	1/40	4.4×10^2	2.5×10^2
	1/80	2.9×10^2	5.0×10^2

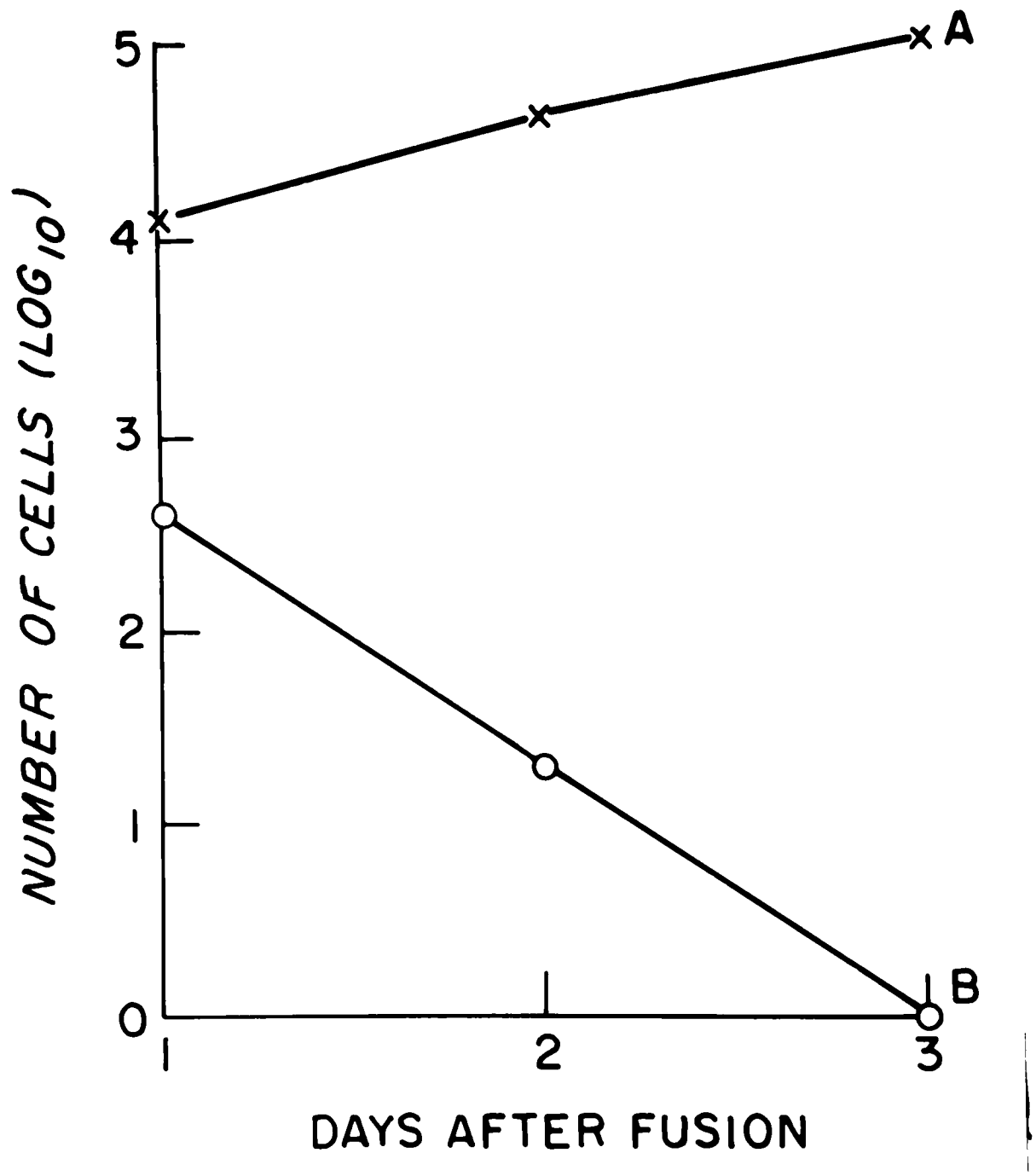

Figure 68. Number of HeLa cells (A) and heterokaryons (B) after fusion of normal HeLa cells and rat lymphocytes.

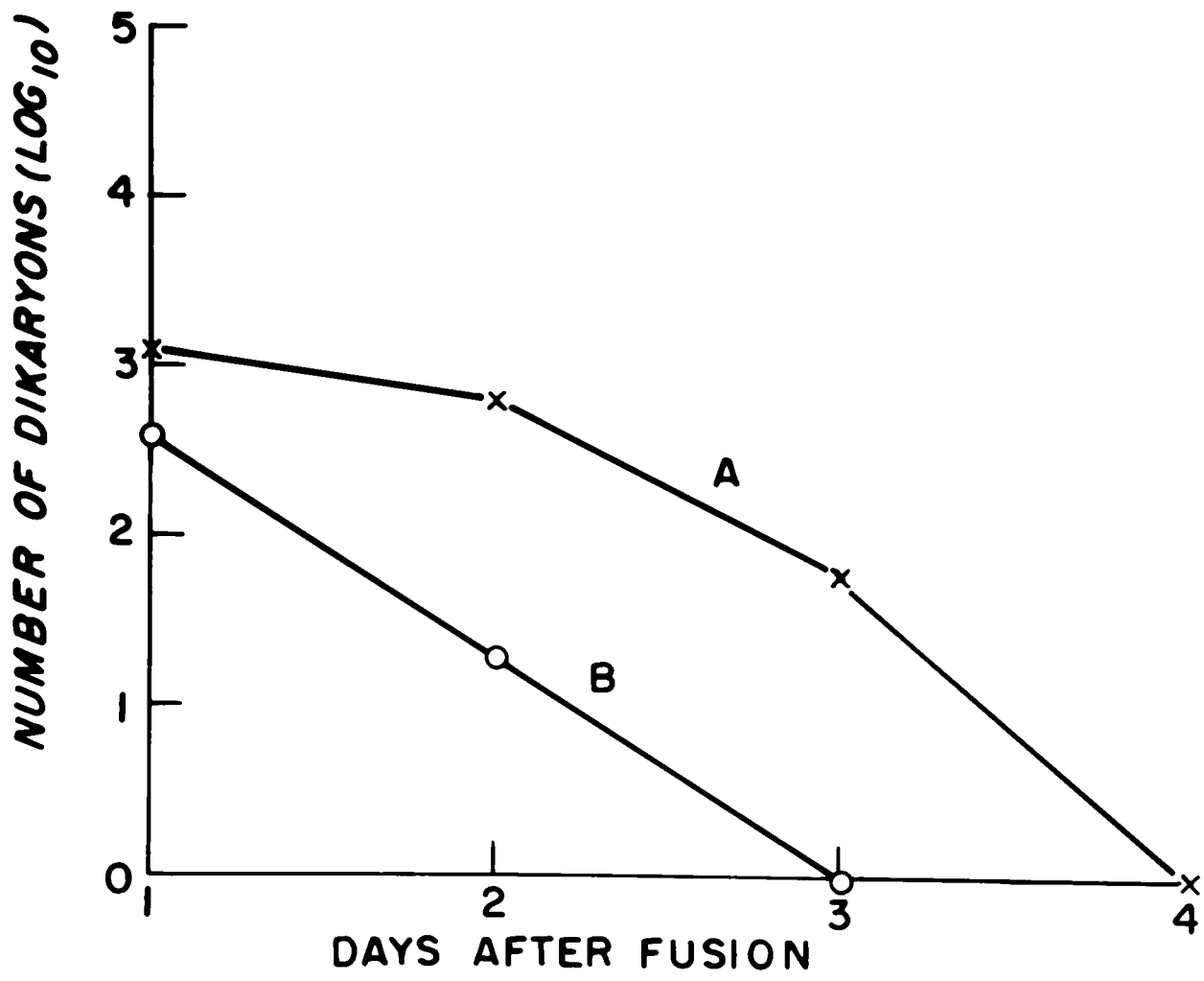

Figure 69. Number of HeLa-rat lymphocyte heterokaryons (dikaryons) after fusion of lymphocytes with gamma-irradiated HeLa cells (A) and normal HeLa cells (B).

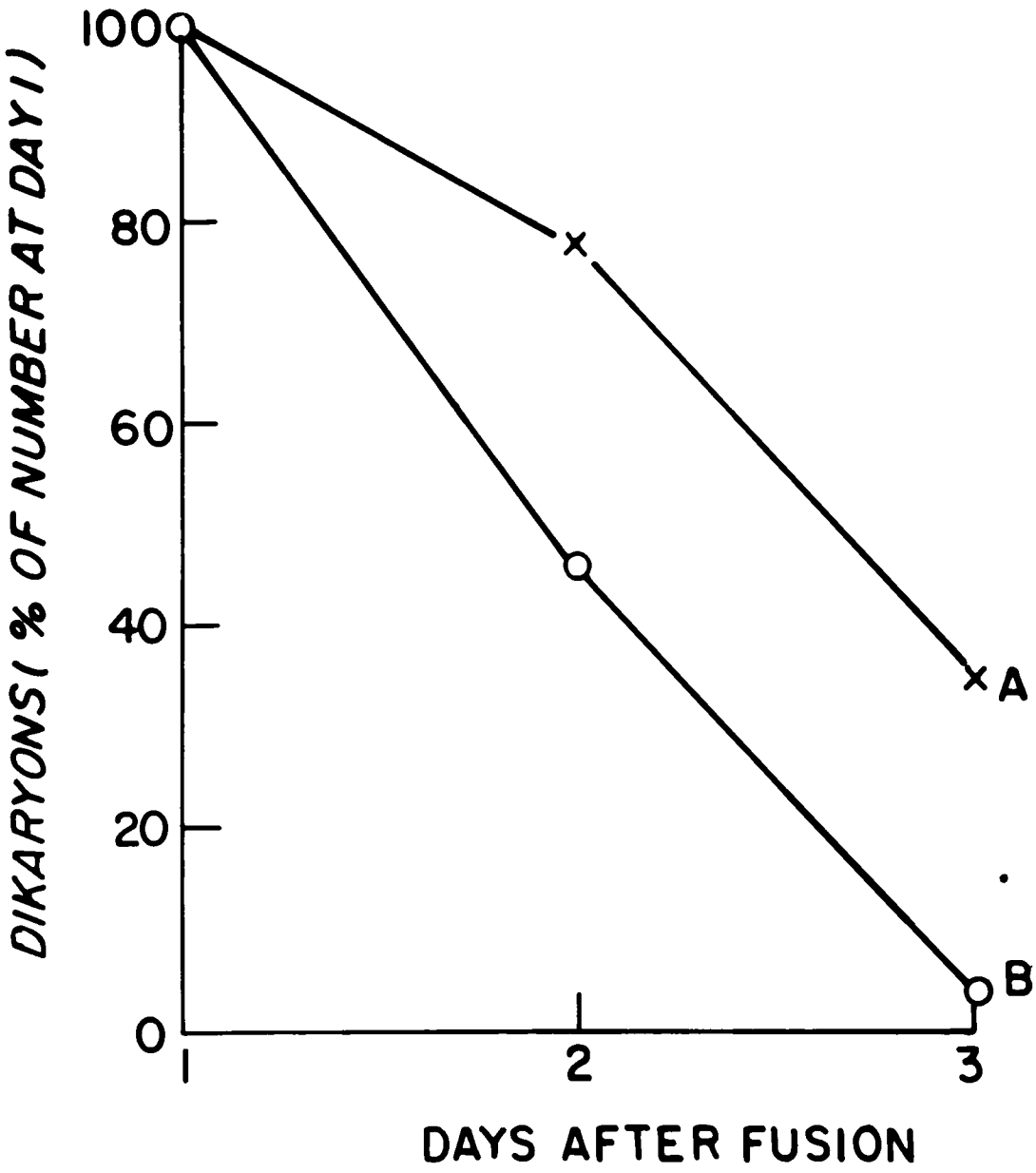

Figure 70. Persistence of dikaryons after fusion of gamma-irradiated HeLa cells with chick embryo erythrocytes (A) and rat lymphocytes (B).

seen (Fig. 67). The results of haemadsorption experiments are shown in Table 17. Although the percentage of labelled dikaryons remained fairly constant, the degree of labelling was reduced and labelling was often patchy.

It was found that these heterokaryons were considerably more resistant to antiserum than single fibroblasts. Table 18 shows the results of an experiment in which 2×10^6 HeLa cells were fused with 3×10^6 fibroblasts with 2,000 HAU of virus.

8. HeLa-Rat Lymphocyte Heterokaryons

There has been some difficulty in obtaining good fusions of HeLa cells and lymphocytes and in removing all unfused lymphocytes from HeLa cells and heterokaryons by differential centrifugation. Some dead lymphocytes often remained adherent to the coverslip or to the surface of single HeLa cells. After the fusion of normal HeLa cells and lymphocytes heterokaryons disappeared quickly (Fig. 68). Usually about 3×10^6 HeLa cells and 5×10^7 lymphocytes were fused with 500 HAU of virus. As in experiments with HeLa-chick erythrocyte heterokaryons survival could be increased by gamma-irradiating the HeLa cells before fusion, as shown in Fig. 69. When lymphocytes or chick erythrocytes were fused with gamma-irradiated HeLa cells each type of heterokaryon disappeared at a similar rate (Fig. 70).

Mixed haemadsorption experiments were carried out with rabbit antiserum to rat lymphocytes. This serum was specific at a dilution of 10^{-3} without absorption and gave good labelling on heterokaryons as shown in

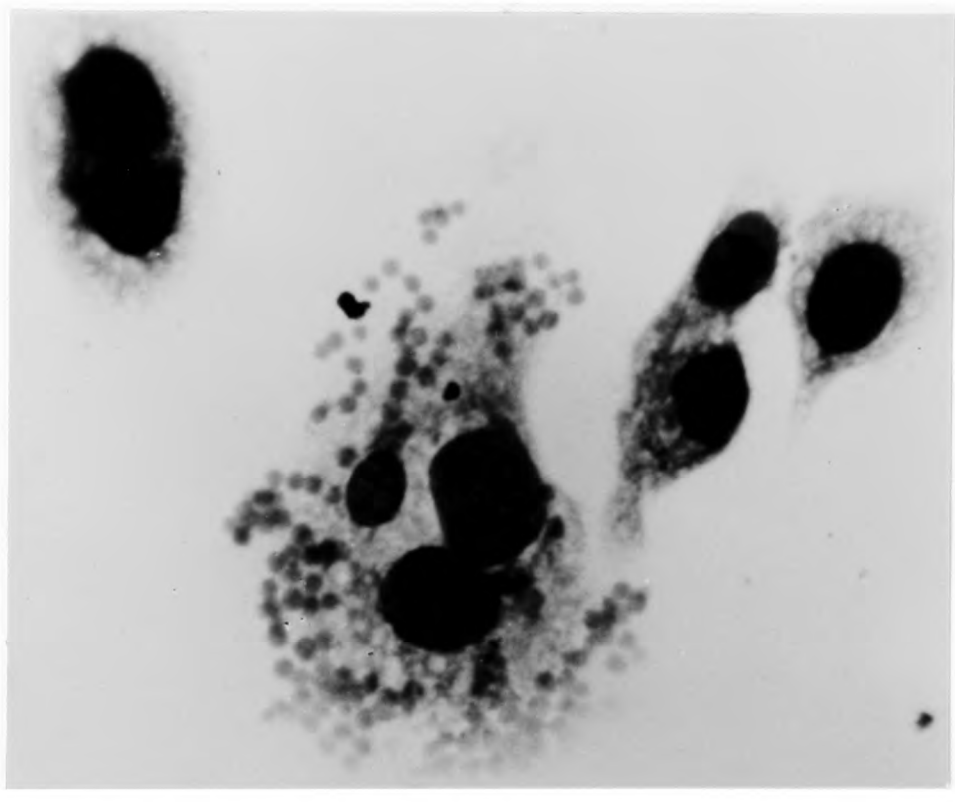

Figure 71. Mixed haemadsorption on HeLa-rat lymphocyte heterokaryon with rabbit anti-lymphocyte serum. Single HeLa cells are unlabelled. x 500.

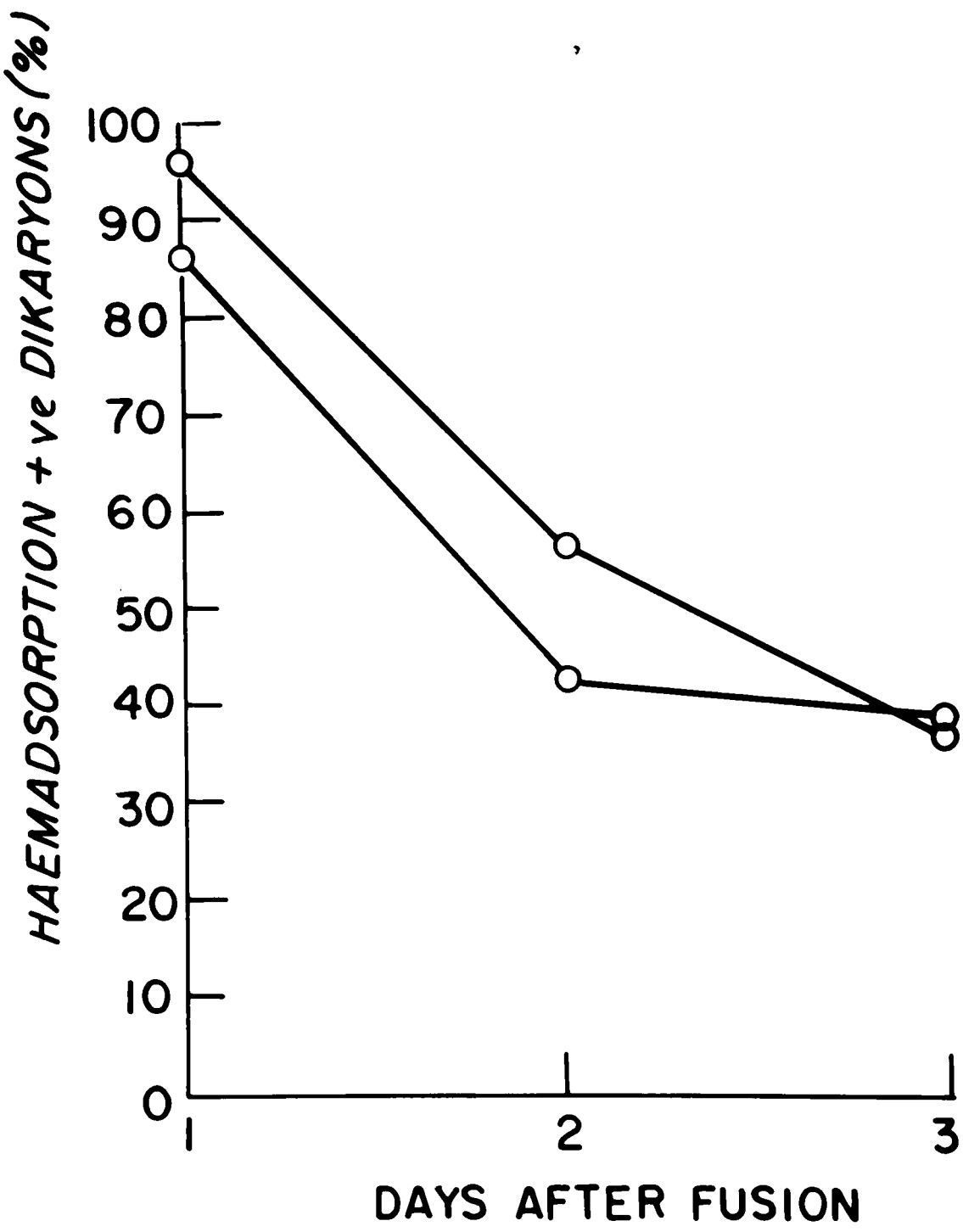

Figure 72. Percent of HeLa-lymphocyte dikaryons labelled for lymphocyte antigens by mixed haemadsorption in two separate experiments.

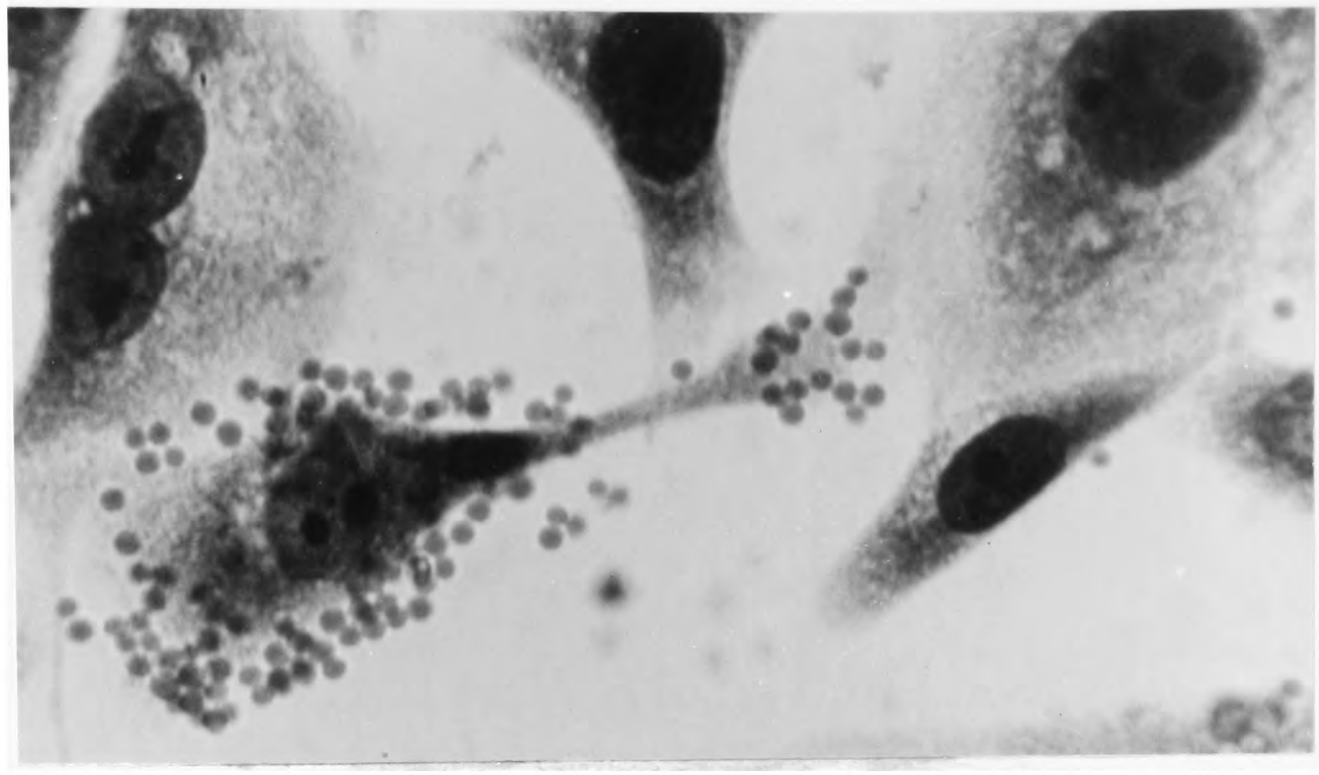

Figure 73. HeLa- rat lymphocyte heterokaryon labelled for lymphocyte antigens by mixed haemadsorption 24 hours after fusion. The lymphocyte nucleus has not yet begun to enlarge. Single HeLa cells are also present. x 720.

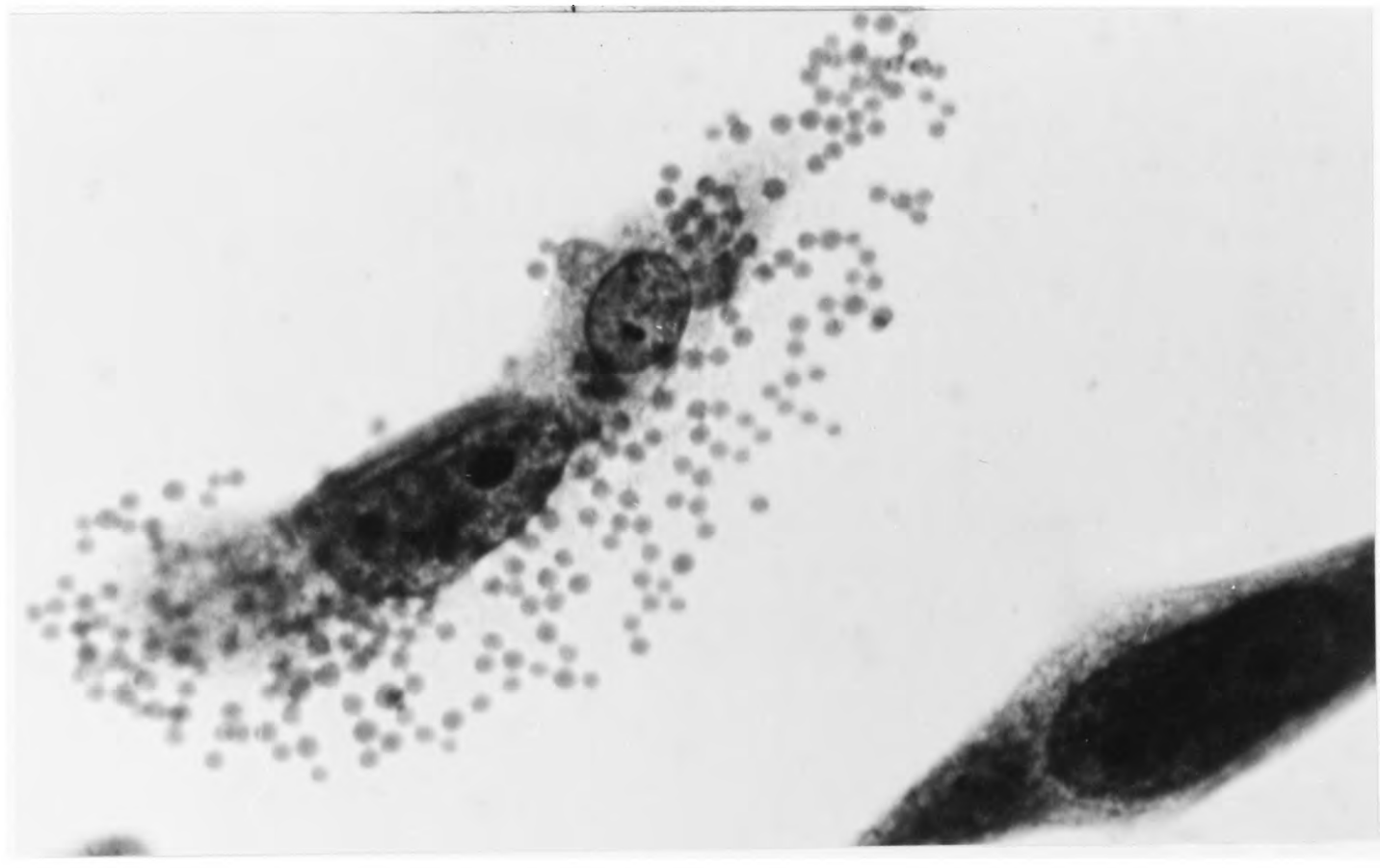

Figure 74. HeLa-rat lymphocyte heterokaryon 3 days after fusion. The nucleus of the lymphocyte has enlarged and contains a small nucleolus and the cell is labelled for lymphocyte antigens by mixed haemadsorption. x 720.

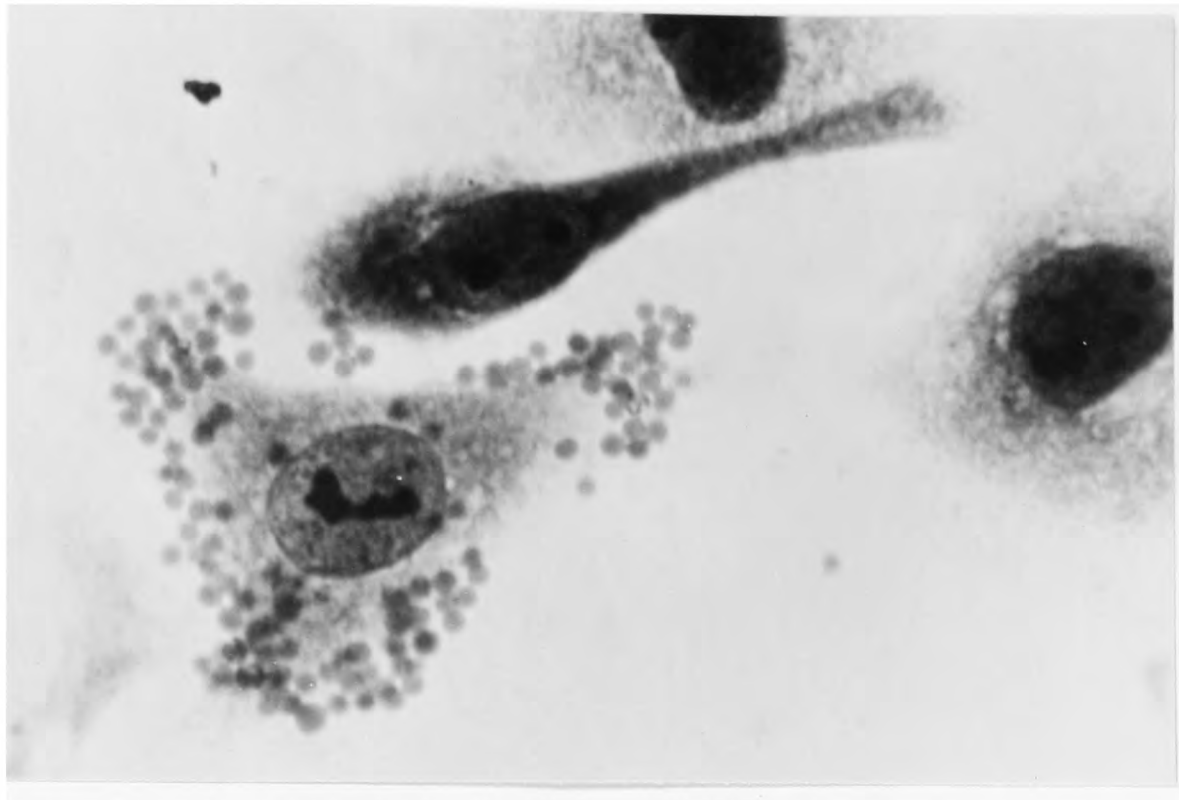

Figure 75. HeLa-lymphocyte heterosynkaryon labelled for lymphocyte antigens 48 hours after cell fusion. x 720.

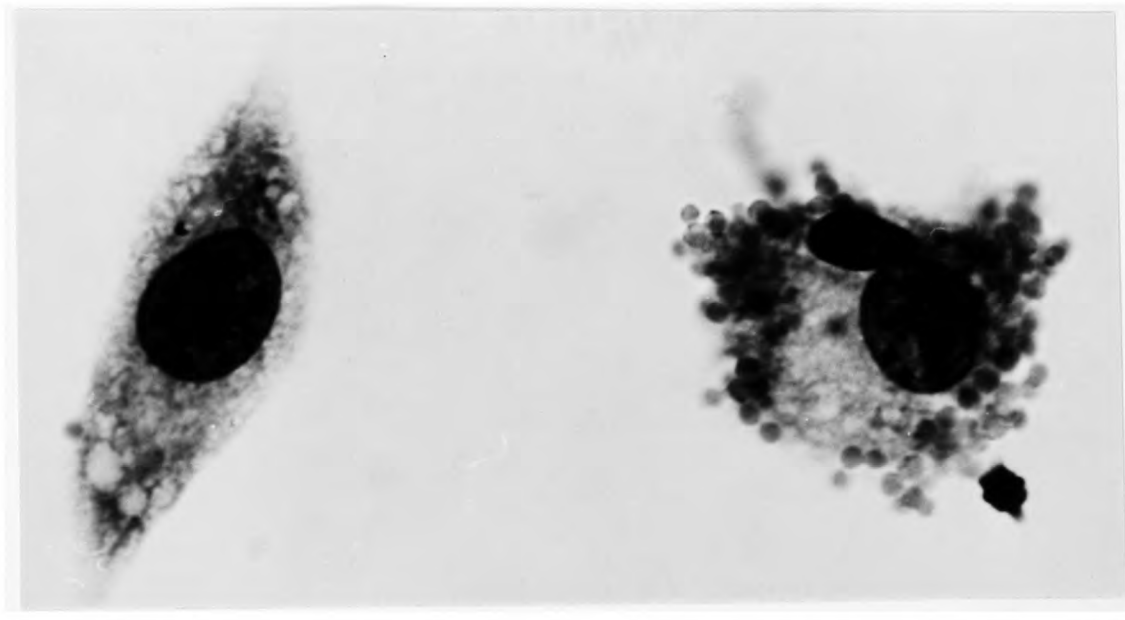

Figure 76. L cell-rat lymphocyte heterokaryon labelled with rabbit antiserum to rat serum 24 hours after fusion. A single HeLa cell is unlabelled. x 720.

Fig. 71. To have enough heterokaryons for counts it was necessary to irradiate HeLa cells before fusion. When this was done antigen disappeared from heterokaryons in the same way as from chick erythrocyte heterokaryons (Fig. 72).

These heterokaryons often showed a considerable amount of movement (Fig. 73) and surface antigen was found on some heterokaryons in which the lymphocyte nucleus was greatly enlarged (Fig. 74). Occasional mononuclear cells were strongly labelled for lymphocyte antigen and may represent heterosynkaryons (Fig. 75). Rabbit antiserum to rat serum (Burroughs Wellcome) was used for mixed haemadsorption in one experiment when lymphocytes were fused with mouse L cells. Most of these heterokaryons were unlabelled but occasional cells showed strong haemadsorption 24 hours after fusion (Fig. 76). No heterokaryons were labelled with this serum 48 hours after fusion.

9. Hamster Fibroblast-L cell Heterokaryons

It was difficult to obtain good mixed haemadsorption on hamster fibroblasts as previously found with chick fibroblasts. Rabbit anti-serum to hamster erythrocytes and fibroblasts did not give 100% labelling. Moreover, hamster embryo cells growing on monolayers in our medium showed considerable variation in morphology and many cells with giant nuclei appeared within a few days of plating. Rabbit anti-hamster fibroblast serum cross-reacted on L cells in mixed haemadsorption experiments. Complete removal of cross-reacting antibodies could be obtained by *in vivo* absorption of this serum in mice as previously described for anti-L

serum. Anti-L serum could be made specific by *in vivo* absorption in hamsters. Mouse or hamster serum titrated for residual rabbit antibody by mixed haemadsorption still gave good labelling although the labelling with rabbit anti-hamster serum on hamster cells remained patchy.

A number of experiments were done in which 3×10^6 L cells and 3×10^6 hamster embryo cells were fused with 2,000 HAU of virus. The antigens of each species were present on heterokaryons but because of patchy labelling with anti-hamster serum and because a double labelling system was not worked out, exact figures were not obtained. It was also difficult to follow heterokaryons because of rapid culture overgrowth by single hamster and L cells. After one fusion, done before any antisera were available, the cells were grown *in vitro* for one month. Haemadsorption done at this time showed that all cells bore mouse antigens only. Neither hybrid cells nor primary hamster embryo cells survived or grew as well as mouse L cells.

10. HeLa-rabbit Macrophage Heterokaryons

2×10^6 rabbit macrophages were fused with 5×10^6 HeLa cells using 1,000 HAU of virus. Macrophage nuclei could be distinguished from HeLa nuclei initially by their small size and also by the absence of nucleoli although small nucleoli do appear after several days in culture. Twenty-four hours after fusion coverslips bearing HeLa-macrophage heterokaryons, macrophages and HeLa cells were rinsed in Hanks' solution and incubated for 15 minutes at $37^\circ C$ in Hanks' solution

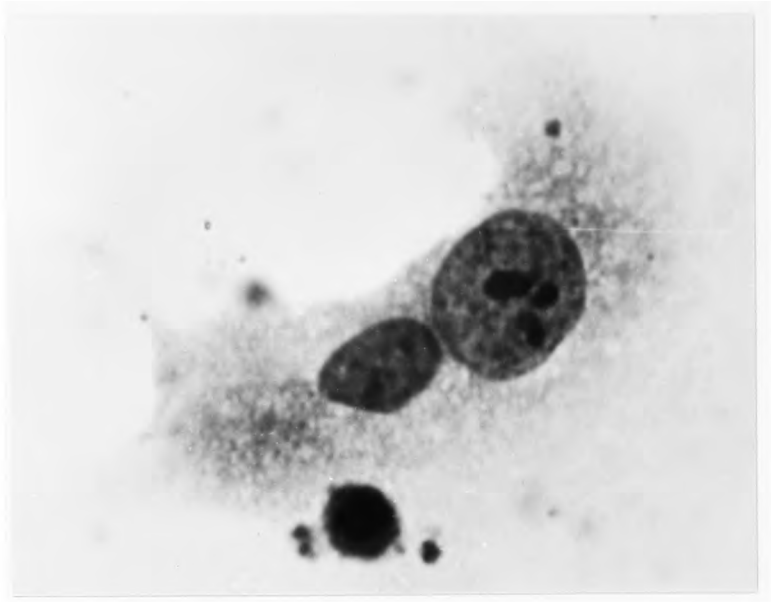

Figure 77. HeLa-rabbit macrophage heterokaryon 24 hours after fusion. It is unaffected by a concentration of leucocidin which has killed the adjacent single macrophage. x 720.

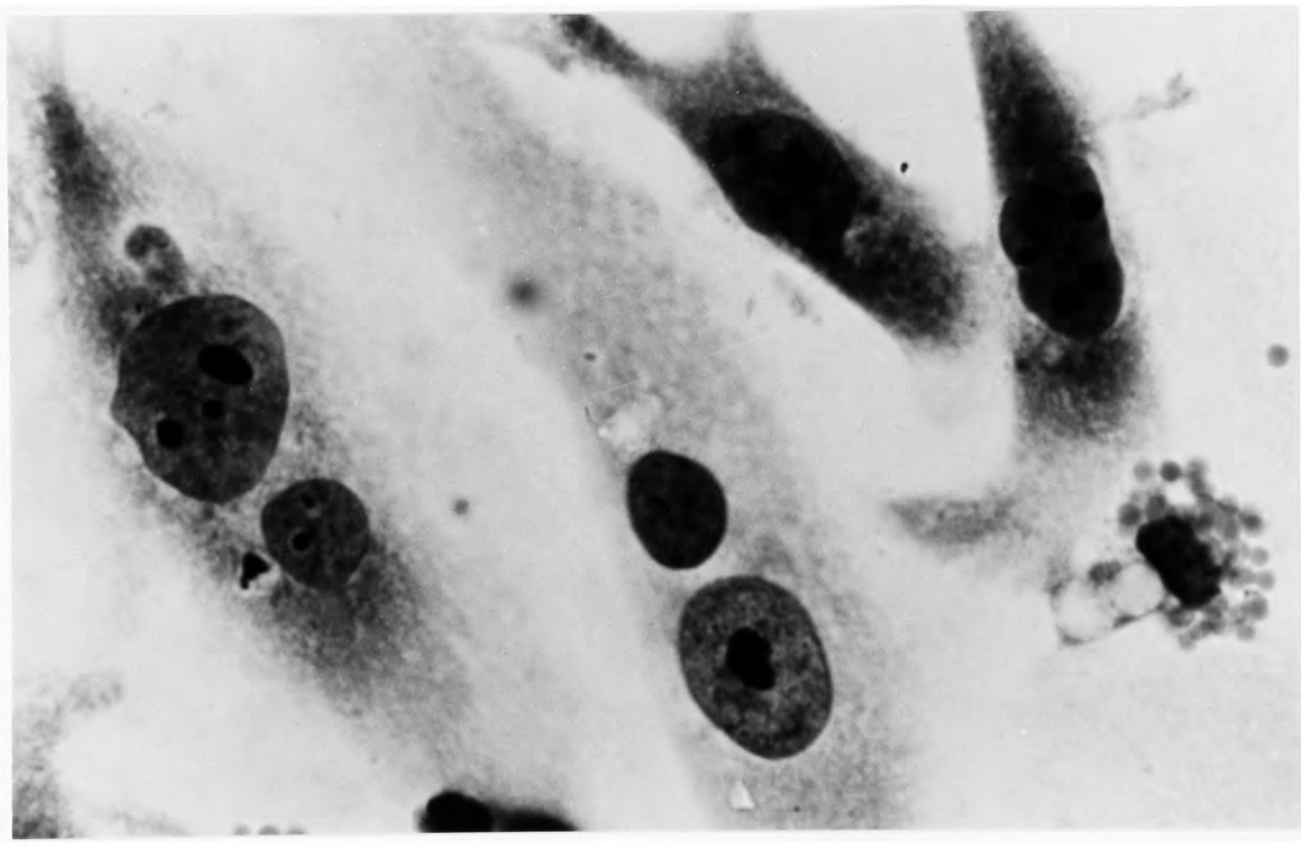

Figure 78. HeLa-rabbit macrophage heterokaryons which have lost the property of adsorbing sensitized erythrocytes shown by the single macrophage. Single HeLa cells are also present. x 720.

Table 19

Effect of Leucocidin on HeLa-macrophage Heterokaryons

Cells	Number of cells per coverslip	
	Control	Leucocidin treated
Macrophages	7.4×10^3	1.6×10^3 (all lysed)
Heterokaryons	2.4×10^2	2.6×10^2

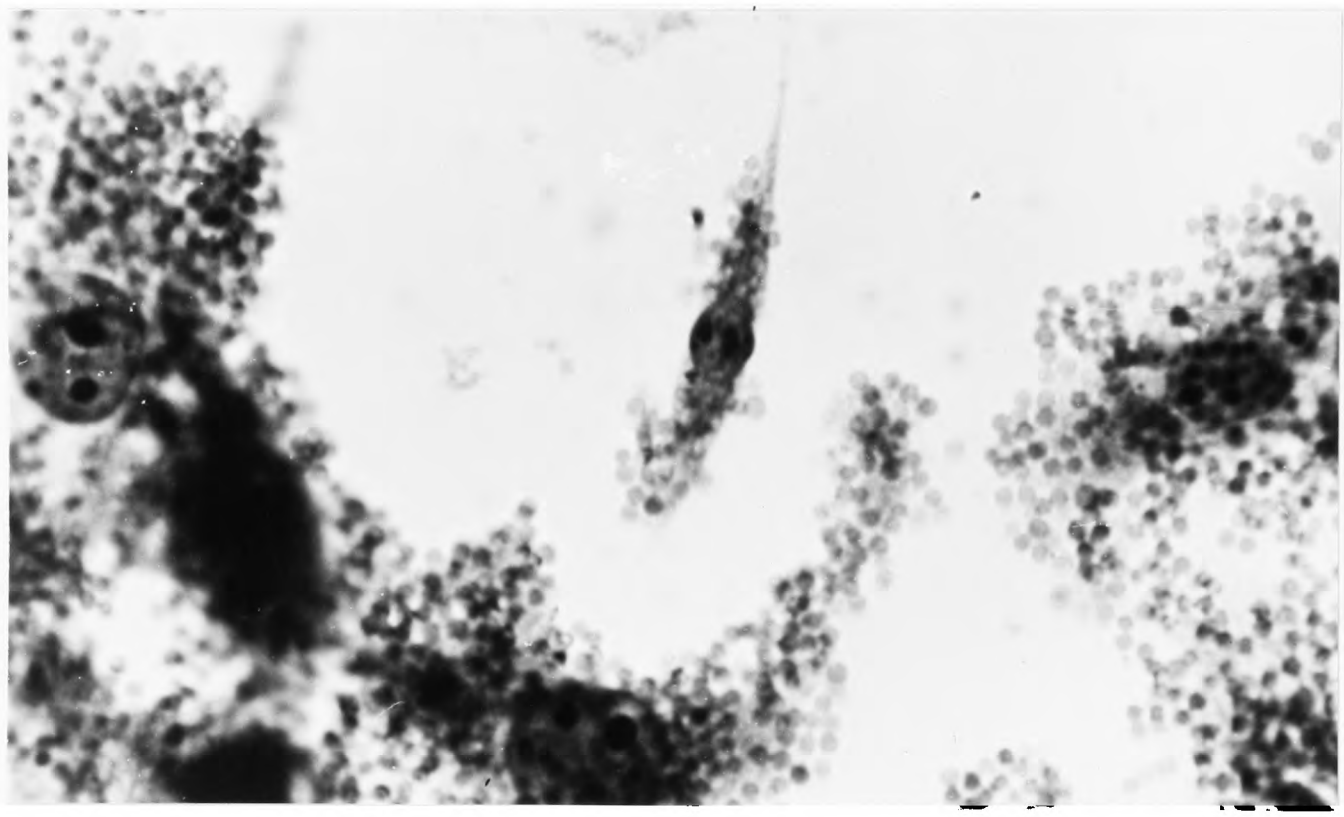

Figure 79. Single L cell (centre) with gamma-irradiated HeLa cells 24 hours after plating. All cells are labelled for HeLa antigens by mixed haemadsorption using specific antiserum. x 600.

containing 100 µg/ml. of leucocidin. Two µg of each of the two components of leucocidin is an excess lethal dose for 10^8 polymorphonuclear leucocytes. After incubation the coverslips were stained and examined. Heterokaryons were not affected by a concentration of leucocidin which killed all single macrophages as shown in Fig. 77. Cell counts are given in Table 19.

These heterokaryons were also checked for the macrophage-type of haemadsorption with doubly sensitised erythrocytes. Not all single macrophages showed this type of haemadsorption 24 hours after fusion, but some did, while no heterokaryons adsorbed the sensitised erythrocytes as shown in Fig. 78.

11. Antigen Transfer between Tissue Culture Cells

Haemadsorption experiments with heterokaryons and mixtures of single cells such as HeLa and L showed that specific antiserum labelled only the appropriate cells. There was no apparent antigen transfer from one type of cell to another. Since some cells were grown on feeder layers it was important to ensure that gamma-irradiated cells did not shed antigen which might be detected on cells of another species. Labelling experiments for HeLa antigen were therefore carried out when 10^4 L cells were grown on coverslips in a Petri dish with 5×10^5 HeLa cells given 5,000 r.

One day after plating occasional single L cells were labelled for HeLa antigen (Fig. 79) although many were unlabelled. Apparently some HeLa antigen can be picked up on the surface of L cells. On subsequent

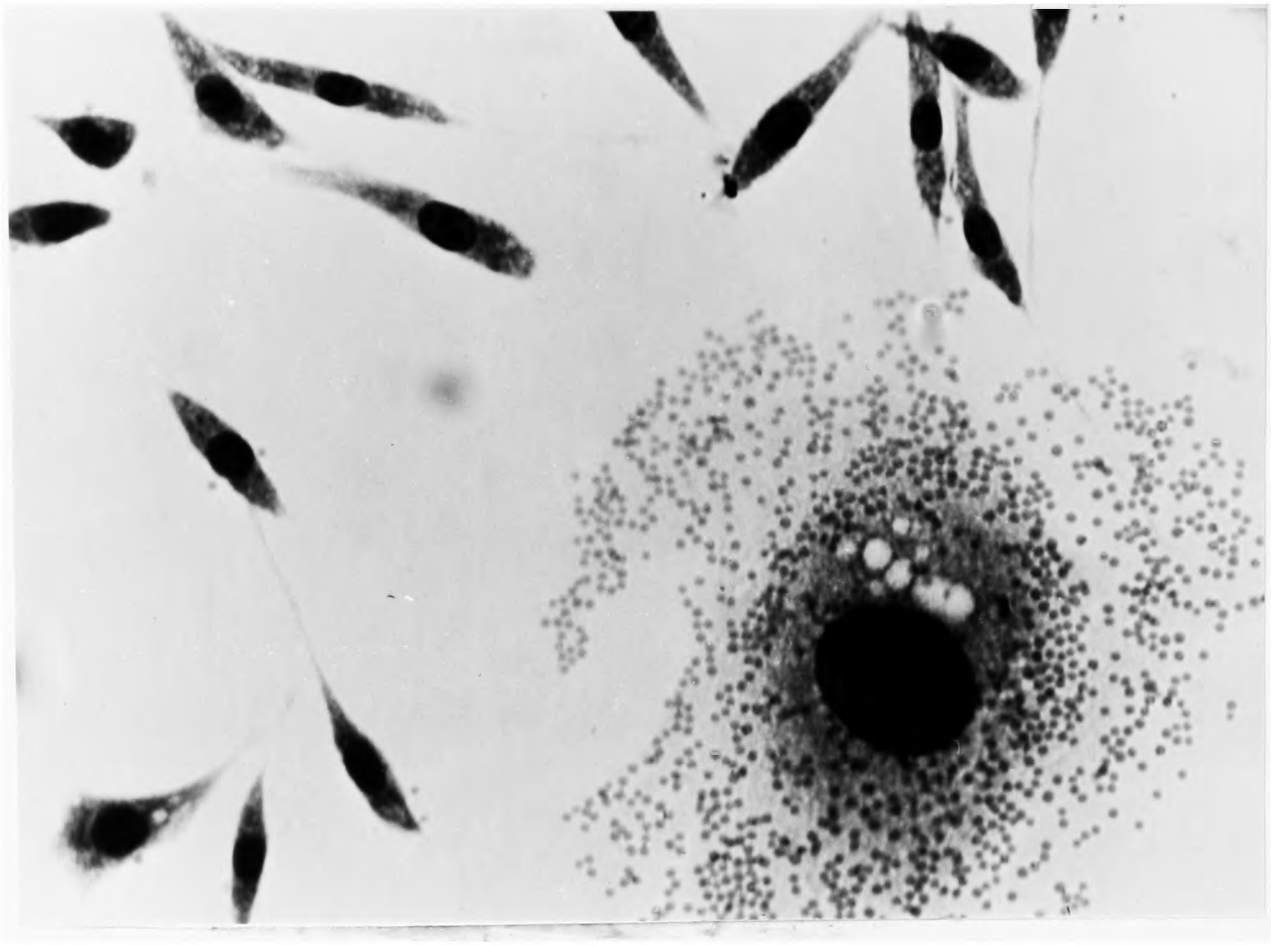

Figure 80. Group of L cells with gamma-irradiated HeLa cell 4 days after plating. Labelling for HeLa antigens is confined to the HeLa cell but extends beyond the visible cell margin., x 600.

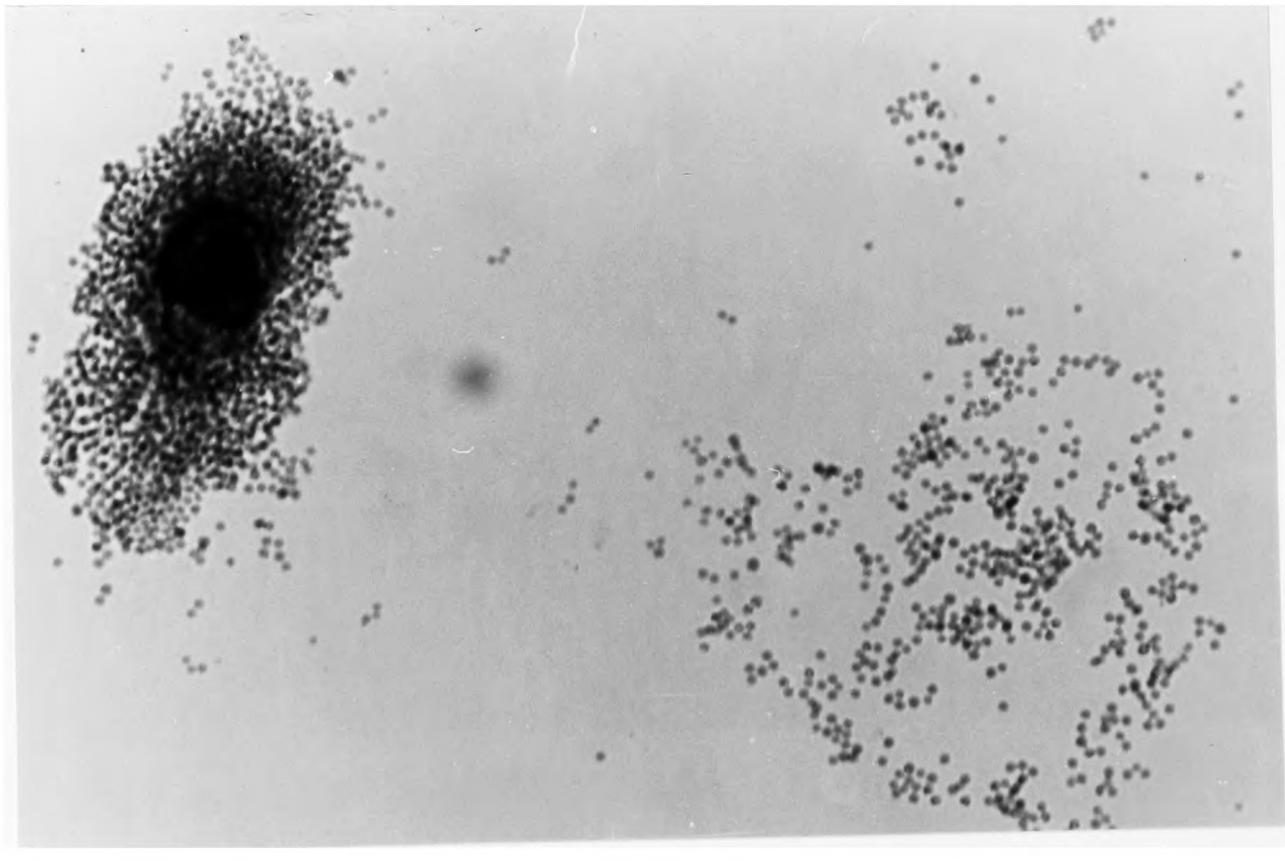

Figure 81. Mixed haemadsorption for HeLa antigens 4 days after gamma-irradiation of HeLa cells. Antigen has remained adherent to the coverslip. x 330.

days most L cells were unlabelled for HeLa antigen (Fig. 80). As colony size increased some haemadsorption for HeLa antigen was observed between closely adjacent L cells. This may have been due to persistence of some HeLa antigen on the glass after the death of gamma-irradiated HeLa cells. This can occur, as seen in Fig. 81, although it seemed to be a rare event with most feeder layer cells.

12. Persistence of Antibody

The persistence of erythrocytes on the surface of growing HeLa cells for several days after mixed haemadsorption suggested that antibody labelling might provide a method for studying surface antigen turnover of tissue culture cells. The first problem was to find an antiserum concentration which would not affect cell growth. 10^6 trypsinised HeLa cells suspended in Hanks' solution were exposed to 1/100 rabbit anti-HeLa serum for 15 minutes at 37°C and then washed three times in 20 ml. of PBS. They were suspended in culture medium and placed in a Petri dish containing coverslips. Twenty-four hours later the number of adherent cells per coverslip was the same for cells exposed to anti-HeLa serum as for those exposed to the same concentration of normal rabbit serum. Haemadsorption with doubly sensitised erythrocytes showed that all HeLa cells were still very strongly labelled with rabbit antibody. The medium was checked for residual antibody by mixed haemadsorption on control HeLa cells and all were labelled. Apparently some antibody elution had occurred. Medium was changed daily and the antibody-labelled cells continued to grow well. Some residual antibody

was detected in the medium 2 days after the initial exposure but none after that, yet all cells were labelled with antibody 5 days after exposure and some were still labelled 8 days later, as shown by the adherence of doubly sensitised erythrocytes.

To test whether antibody disappearance was due to the degradation of antibody globulin, rabbit anti-HeLa serum diluted 1/1,000 in Hanks' solution was incubated at 37°C, and titrated for mixed haemadsorption after 1 and 2 weeks of incubation. There was no significant reduction in the haemadsorption titre of the antiserum and so it appeared that antibody disappearance could not be explained by degradation.

Although antibody was not detected in the medium it was still important to rule out antibody transfer between adjacent cells or between adjacent antigens on the same cell, since this would greatly prolong antibody persistence. To test this possibility HeLa cells were labelled with [^{3}H]-thymidine for 24 hours, as described in Materials and Methods. They were then harvested with trypsin, exposed to 1/100 anti-HeLa serum, and washed. 5×10^5 of these cells were mixed with 5×10^5 control HeLa cells unexposed to either [^{3}H]-thymidine or anti-HeLa serum. After 24 hours of growth on coverslips, a combination of mixed haemadsorption (with doubly sensitised erythrocytes only) and autoradiography showed that many cells which were not labelled with [^{3}H]-thymidine had adherent erythrocytes. Therefore some antibody transfer between adjacent cells had taken place, at least within the first 24 hours of antibody labelling.

Several different methods were used in an attempt to prevent antibody transfer.

(i) Growth of HeLa cells on L cell feeder layer

10^4 antibody-labelled HeLa cells were grown in a Petri dish with a feeder layer of 5×10^5 gamma-irradiated L cells and the medium changed daily. All HeLa cells were strongly labelled for rabbit antibody for 2 days after exposure and there was a considerable amount of labelling until 4 days after exposure. There was enough separation between single HeLa cells to prevent antibody transfer but antibody transfer on the same cell was not definitely eliminated. At least in short experiments with erythrocytes antibody transfer from site to site on the same cell is considered to be much more rapid than antibody transfer from cell to cell (Weinrach and Talmage, 1958).

(ii) Use of guinea pig and rabbit antisera

Cells were labelled with 1/100 guinea pig anti-HeLa serum and then grown in medium containing 1/500 rabbit anti-HeLa serum. It was hoped that if any guinea pig antibody did elute from the cell surface its place would be taken by rabbit antibody and thus antibody transfer would be prevented. Erythrocytes doubly sensitised to adhere to guinea pig globulin had rabbit antibody as the external layer and so would not detect rabbit antibody on the cell surface. However, the experiment could not be done in reverse using HeLa cells labelled with rabbit antibody and incubated in medium containing guinea pig antibody since erythrocytes doubly sensitised to adhere to rabbit globulin, with sheep anti-rabbit globulin as the external layer, will also adhere weakly to guinea pig globulin.

To test this method 5×10^5 HeLa cells labelled with [^{3}H]-thymidine

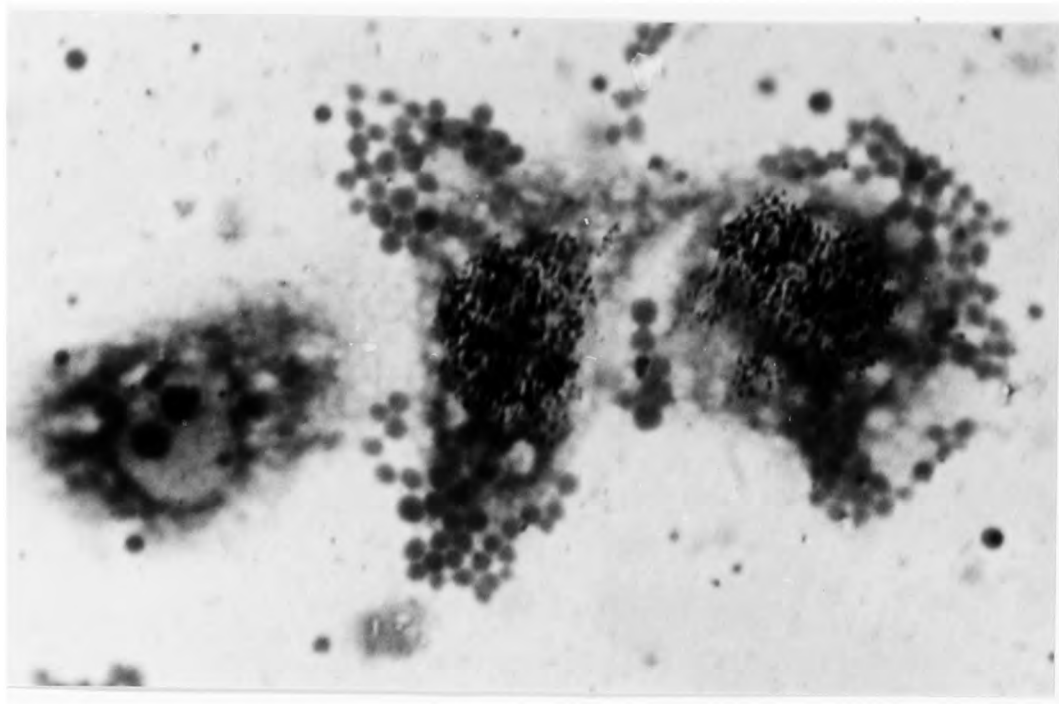

Figure 82. Mixed haemadsorption on HeLa cells labelled with guinea pig anti-HeLa serum and [^{3}H]-thymidine 24 hours earlier. The single unlabelled HeLa cell shows that guinea pig antibody was not transferred to adjacent cells. The cells were grown in rabbit anti-HeLa serum. x 720.

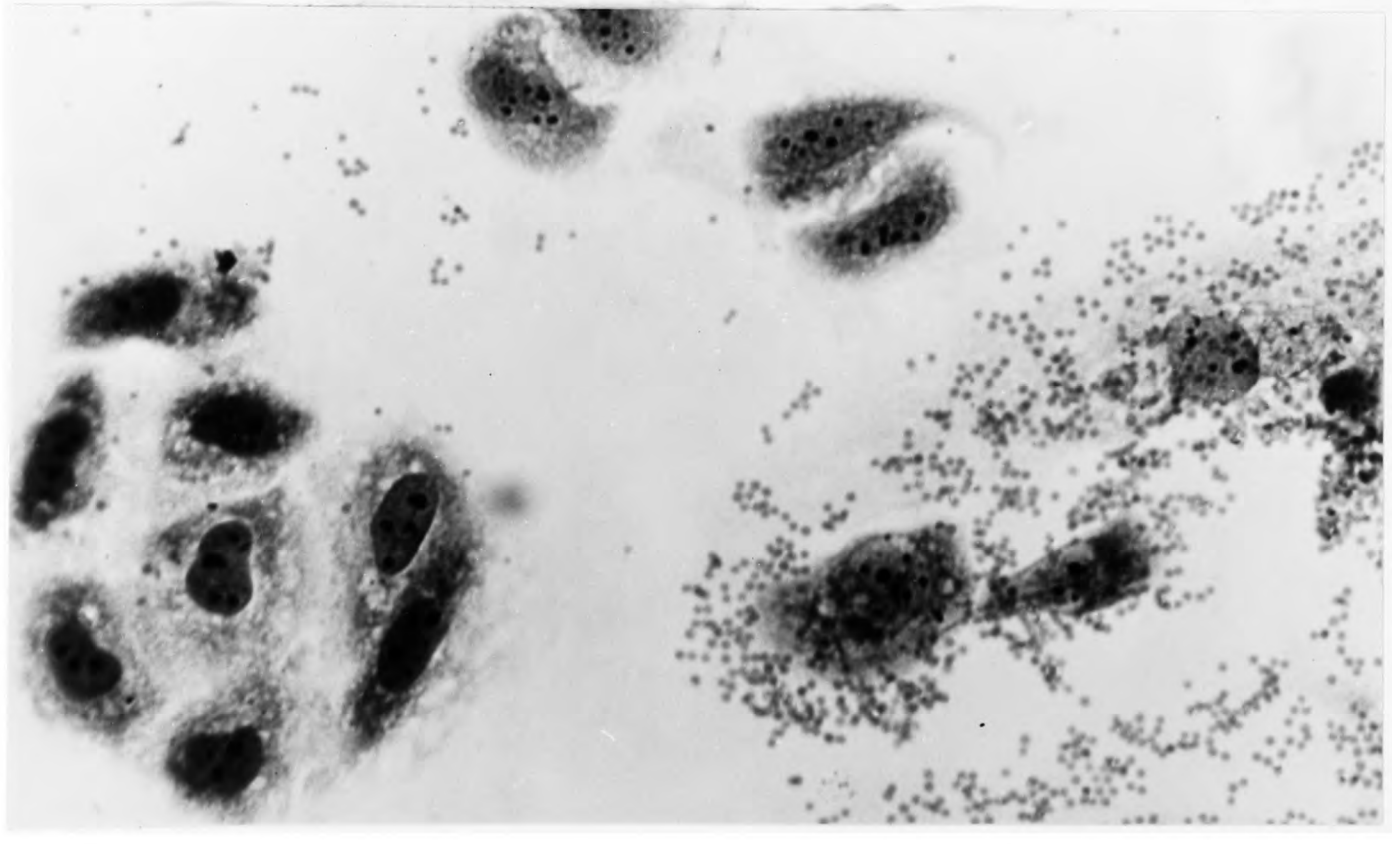

Figure 83. Mixed haemadsorption on HeLa cells 2 days after exposure to anti-HeLa serum at a dilution of 1/1,000. Multiplying HeLa cells are mainly unlabelled while gamma-irradiated cells are strongly labelled. x 330.

and guinea pig antiserum were mixed with 5 x 10^5 control HeLa cells in medium containing 1/500 rabbit anti-HeLa serum. Autoradiography and mixed haemadsorption done 24 hours later showed that there was very little transfer of guinea pig antibody, at least to adjacent cells (Fig. 82). However, there was no remaining guinea pig antibody 48 hours after exposure. This could indicate surface antigen turnover or simply displacement of guinea pig globulin by the excess of rabbit antibody.

An experiment was also set up to see if antibody disappeared from normal and gamma-irradiated HeLa cells at different rates. 1.8 x 10^3 normal HeLa cells were mixed with 5 x 10^5 HeLa cells previously given 6,000 r of gamma-irradiation and the cells grown for 24 hours in a 60 mm. Petri dish containing coverslips. Coverslips were then exposed to 1/1,000 rabbit anti-HeLa serum in Hanks' solution for 15 minutes and washed thoroughly in PBS. Twenty-four hours later all HeLa cells had adherent antibody as shown by mixed haemadsorption. There was no residual antibody detectable in the medium but the medium was nevertheless changed. Forty-eight hours after exposure to antiserum all cells showed some degree of labelling by mixed haemadsorption but the growing HeLa cells were much more weakly labelled than adjacent gamma-irradiated cells, as shown in Fig. 83. Three days after antibody labelling, the groups of growing HeLa were mainly unlabelled while most of the remaining irradiated cells were still strongly labelled.

DISCUSSION

Each type of heterokaryon will be discussed separately since individual cell combinations have their own peculiarities. Generalisations will be made at the end of this Discussion and related to the objectives stated in the Introduction.

1. Detection of surface antigens

Mixed haemadsorption proved to be a sensitive and effective technique for labelling surface antigens of monolayer cells. Erythrocytes were not lost during fixation and staining. Mixed agglutination and immune adherence were not as effective for cells on a glass surface but these techniques were not investigated in any detail. It is probable that they could be adapted for the study of heterokaryons, and mixed agglutination could be particularly useful for following Forssman or blood group antigens. In the present experiments mixed agglutination was obtained only with high concentrations of antiserum. There are several possible reasons for inadequate labelling. Erythrocytes were lost during the washing, fixation, and staining of coverslips. Mixed agglutination may not be as effective on monolayer cells as on cells in suspension, although Franks and Dawson (1966) did obtain good labelling on unfixed monolayer cells with human immune anti-A serum. In addition, antisera produced to tissue culture or ascites cells may not be as effective for demonstrating species antigens by mixed agglutination as anti-erythrocyte sera (Boyle and Davies, 1966) and in my experiments

antiserum to erythrocytes was not used for mixed agglutination. With anti-erythrocyte serum Coombs et al. (1961a) were able to obtain mixed agglutination between HeLa cells and human erythrocytes in suspension to a dilution of 1/1,280. Very recent experiments have used mixed agglutination in suspension to demonstrate human and mouse species antigens on a human-mouse hybrid cell line (Weiss and Green, 1967).

Immune adherence is particularly useful in demonstrating complement fixation on tissue culture cells as shown by Spooner and Sell (1966) and Sell and Spooner (1966) but in studies on surface antigens there is danger of damaging cells with high concentrations of antibody and complement.

2. HeLa-Ehrlich heterokaryons

(i) Surface antigens

Mixed haemadsorption studies show that there is rapid and even mixing of the surface antigens of each cell type. Surface antigens must be capable of movement in relation to each other and mixing may be assisted by the constant activity of the cell surface which was discussed in the Introduction. The persistence of species antigens on cells in tissue culture has also been discussed in the Introduction. It is of interest that both Ehrlich and HeLa antigens are expressed on the surface of heterokaryons but this does not enable one to say that there is antigen synthesis and replacement. Protein synthesis is going on in heterokaryons as shown in these and previous studies (Harris and Watkins, 1965) but this does not in itself indicate antigen turnover. Hybrid cells do

not divide more than 4 or 5 times in several weeks and each antigen could simply persist from the time of fusion. Heterokaryons are metabolically active soon after fusion but have a limited life span of 2 or 3 weeks and may not be healthy cells. Antigen loss, for example, of Ehrlich antigen(s) may require not only the absence of antigen synthesis by the Ehrlich component but also active replacement by the HeLa component.

It is known that more than one week after cell fusion many heterokaryons synthesise DNA poorly in comparison to single HeLa cells (Harris and Watkins, 1965) and at this time some heterokaryons appear vacuolated and abnormal. Dead cells can remain adherent to glass for some days after the action of antibody and complement (Oda and Puck, 1961; Weiss, 1965) or after being pricked by a microneedle (Munroe and Daniell, 1965). Therefore Ehrlich antigen could persist on some heterokaryons because they are dead or dying but have remained adherent to coverslips.

One must recognise the faint possibility that growing hybrid cells could lose Ehrlich antigen and would then be undetectable by mixed haemadsorption techniques. However, this seems most unlikely since all cells in these experiments capable of continued growth showed typical HeLa morphology.

(ii) UV-irradiation

Ehrlich antigen could not be persisting on heterokaryons produced with UV-irradiated Ehrlich cells simply because of metabolic inactivity by the HeLa component. During the first few days after cell fusion these heterokaryons show active protein synthesis. HeLa nuclei synthesise

RNA although the amount may not be as great as in heterokaryons produced with unirradiated Ehrlich cells. In spite of the absence of RNA synthesis in many Ehrlich nuclei after UV-irradiation, Ehrlich antigen persists on these heterokaryons. There are several possible explanations for this.

Ehrlich antigen could still be produced in spite of the failure of the Ehrlich nuclei to synthesise RNA. Studies on a variety of enucleate and actinomycin-treated cells and organisms have shown that protein synthesis may continue for hours or even days in the absence of RNA synthesis (reviewed by Harris, 1968).

Also, cell membrane turnover in these non-multiplying heterokaryons may be too slow to result in replacement of all Ehrlich antigen during the period that these heterokaryons remain adherent to glass. Mixed haemadsorption undoubtedly detects many different antigens on the Ehrlich cell surface. Only one such antigen must remain for the heterokaryon to be labelled for Ehrlich antigen. Since Ehrlich and HeLa cells are similar in size the proportion of Ehrlich antigen in or on a heterokaryon is high. More Ehrlich antigen may be found in the convolutions of the cell surface which have been inferred from cell electrophoresis studies (Nordling and Mayhew, 1966; Patinken and Doljanski, 1965). Moreover there may be an intracellular pool of Ehrlich membrane components (Cook, Laico and Eylar, 1965). The whole question of cell membrane turnover is complex and will be discussed in more detail in association with the disappearance of hen antigen from HeLa-hen erythrocyte heterokaryons.

It is not certain why there is evidence of Ehrlich antigen disappearance only when UV-irradiated Ehrlich cells are fused with gamma-irradiated HeLa cells. The use of gamma-irradiated cells for fusion experiments will also be discussed with HeLa-hen erythrocyte heterokaryons. The actual effect of UV-irradiation on cells is not well understood. It seems to be efficient in producing gene mutation but unlike x-irradiation produces few actual chromosome breaks (Lee and Puck, 1960). These workers found that after a UV dose of 554 ergs/mm^2 only 1.4% of HeLa cells survived as measured by subsequent colony formation. This is far lower than the doses of 5,000 to 50,000 ergs/mm^2 given to Ehrlich cells. UV is known to be efficient in damaging the mitotic spindle (Carlson and Hollaender, 1948).

(iii) Adhesion to glass

Since the mechanism of cell adhesion is not well understood, it is difficult to explain why these heterokaryons, formed with non-adherent Ehrlich cells, stick and spread out so well. The persistence of the HeLa surface property of adhering to glass indicates, as did antigen studies, that the HeLa surface is not covered by any mucopolysaccharide or protein layers derived from the surface of Ehrlich cells. Adherence does not seem to be dependent on a definite concentration of sites at the cell surface since heterokaryons formed by the fusion of a few HeLa cells with many Ehrlich cells will still stick. On the other hand, the degree of mixed haemadsorption with weak anti-Ehrlich serum was dependent on the ratio of Ehrlich to HeLa cells in heterokaryons. It is possible that a controlled shearing force, for example the method of

Weiss and Kapes (1966), would show that heterokaryons with a high proportion of Ehrlich nuclei are most easily detached.

(iv) Morphology and movement

The fibroblastic morphology of these cells, a mode of growth unlike either parent, is difficult to explain, as are the long cytoplasmic projections of some heterokaryons. Yerganian and Nell (1966) described hamster fibroblast hybrids produced with UV-inactivated Sendai virus which had a morphology intermediate between the 2 parents. Some of these heterokaryons had long cytoplasmic processes. Such cytoplasmic connections have been seen in cultures of transformed cells (Bendich, Vizoso and Harris, 1967) but their significance is obscure. As discussed in the Introduction there is no definite explanation for differences in cell morphology although the work of Danielli et al. (1955) with amoebae suggested that morphology was determined by the cytoplasm and cytoplasmic fragments of human cells can stick to glass and even show movement and pinocytosis (Goldstein et al., 1960). The interaction in heterokaryons of cells with differing membranes and cytoplasm seems enough to give rise to an unusual pattern of cell morphology and movement. However, this pattern is dependent on healthy, metabolically active heterokaryons since the first response of many heterokaryons to abnormal cultural conditions is the withdrawal of cytoplasmic processes and subsequent rounding up on the glass. The direct contribution of the nucleus to cell shape is uncertain although the nucleus is of course essential for prolonged viability. Chambers and Fell (1932) showed that cytoplasmic protrusions of a binucleate cell might be withdrawn

after puncture of the nearest nucleus but that movement of the whole cell was later restored, apparently under the direction of the remaining live nucleus. It is not known if Ehrlich and HeLa nuclei influence the projection of cytoplasmic processes from heterokaryons.

(v) Failure to multiply

Although there is antigen mixing at the surface of heterokaryons a phenomenon like allogeneic inhibition in which contact with foreign histocompatibility antigens is sufficient to suppress cell growth seems unlikely, since viable hybrids have been produced *in vitro* with mouse cells carrying different H-2 antigens (Spencer *et al.*, 1964). Antigenic studies have not yet been reported on the interspecific rat-mouse hybrids described by Weiss and Ephrussi (1966a) or the hamster-mouse hybrids described by Davidson *et al.* (1966) but these cells will probably be found to carry at least some species antigens of each parent. Recently Weiss and Green (1967) used mixed agglutination to show human and mouse antigens on a viable hybrid line produced between established mouse cells and diploid embryonic fibroblasts from human lung. It therefore seems unlikely that the mixing of surface antigens could in itself explain poor heterokaryon growth. The multinucleate state seems to prevent the division of most heterokaryons and homokaryons (Harris and Watkins, 1965) and the inability of single Ehrlich cells to grow in vitro is an additional factor (Harris *et al.*, 1966).

(vi) Cytolysis

It is difficult to design experiments which measure precisely the degree of cytolysis of heterokaryons with antibody and complement,

There is considerable variation in the degree of cellular fusion from experiment to experiment and the heterokaryons are more sensitive to alterations in cultural conditions than are normal cells so that prolonged incubation in antibody and complement solutions is impossible. The determination of cytolysis on coverslips is also inexact. In spite of these valid criticisms, however, cytolysis experiments would appear to support the concept that both human and mouse antigens are present on the surface of heterokaryons and that they are intimately mixed.

The fact that HeLa-Ehrlich heterokaryons could be lysed by anti-HeLa and anti-Ehrlich serum agrees with mixed haemadsorption studies which show each antigen. It is a point of interest that heterokaryons were more resistant to the effect of anti-Ehrlich serum than monolayers of mouse L cells, although the fact that our Ehrlich cells cannot be grown on monolayers and used for comparison makes interpretation of results more difficult. The heterokaryons were almost as susceptible to anti-HeLa serum as single HeLa cells although the occasional heterokaryon was more resistant. In spite of the presence of each type of antigen over the surface of heterokaryons as shown by mixed haemadsorption the HeLa surface property of sticking to glass is dominant. In cytolysis experiments also the HeLa antigen seems to be dominant.

It is not certain why some heterokaryons remained unlysed with high concentrations of anti-Ehrlich serum. Intimate mixing of Ehrlich and HeLa antigens may result in anti-Ehrlich antibody molecules being too far apart for complement fixation. Antisera used in these experiments were not analysed for antibody types but after repeated injections

would probably contain mainly IgG antibody. It has been suggested that in some cases IgM antibody may not be very effective in lysing nucleated cells (Sell and Spooner, 1966; Spooner and Sell, 1966) and that for cell lysis by IgG antibody, 2 molecules must be adjacent for complement fixation (Weinrach and Talmage , 1958; Borsos and Rapp, 1965; Humphrey and Dourmaskin, 1965). It is possible that separation of Ehrlich antigens by HeLa antigens may prevent complement fixation and make the heterokaryons more resistant to anti-Ehrlich antibody. However, such an explanation should work in reverse with the heterokaryons being more resistant to anti-HeLa serum than they seem to be.

The fact that combinations of antisera are more effective in producing cytolysis than a single antiserum at the same concentration also suggests that there is intimate mixing of antigens. This interpretation is supported by recent experimental evidence. Moller and Moller (1962) used isoantisera to mouse lymph node and tumour cells to show that the cytotoxic sensitivity was closely related to the concentration of surface antigens. Tumour cells which were resistant to a single iso-antiserum could be lysed by mixtures of antisera. Winn (1962, 1965) also supports the view that cytotoxicity is strongly influenced by the concentration and distribution of antigens on the surfaces of cells. Moller (1963) showed that the cytotoxic sensitivity of mouse embryo cells increased after birth and was correlated with the concentration of H-2 antigens. Further studies by Sanderson (1964) with isoantisera to hybrid lymphocytes and by Moller and Eklund (1965) with antisera to ABO and Rh antigens on human lymph node cells showed that

combinations of antisera could produce more effective cytolysis than a single antiserum. It would appear that the same sort of antigenic mixing is involved in the cytolysis of heterokaryons, although the antisera to whole cells undoubtedly contain antibodies to a great many surface antigens.

(vii) Gamma-irradiated HeLa cells

It appears that normal Ehrlich cells, though they themselves do not stick to glass or grow *in vitro*, make a contribution to the adherence of these heterokaryons since single irradiated HeLa cells and HeLa homokaryons come off the coverslips long before the heterokaryons. The nature of this process, which may involve some degree of repair of radiation damage in the HeLa cells, is not understood. It is even more difficult to explain why these heterokaryons persist for many days after irradiation of both HeLa and Ehrlich cells.

3. HeLa-L heterokaryons

The general process of antigen mixing at the surface of heterokaryons is again illustrated with this cell combination. It is, however, difficult to explain why these heterokaryons show even less evidence of division than HeLa-Ehrlich heterokaryons. One might expect the opposite since L cells grow well *in vitro* while Ehrlich cells do not. The basic incompatibility may lie in the difference between epithelial and fibroblastic cell types rather than in the differences between species.

Experiments with this combination of cells also showed that mixed haemadsorption can be used for the selection of antigenically different

cells which form a very small proportion of a population, since mixed haemadsorption with dilute antiserum did not affect cell viability. However, it has not yet been possible to apply this technique to the selection of a hybrid cell line. Such antigenic selection could be combined with the Puck cloning procedure on feeder layers. One clone bearing the desired antigen could be quickly and easily selected from thousands of unlabelled clones by mixed haemadsorption. This would eliminate the need for microscopic examination of each clone which could be necessitated by morphological similarities between single and hybrid cells.

4. Ehrlich-L heterokaryons

In vivo absorption of rabbit anti-L serum in normal mice has been shown to remove antibodies reacting with Ehrlich cells but leave some antibody which reacts with L cells. This antigen was still present on Ehrlich-L cell heterokaryons. The inability to obtain an antiserum specific for Ehrlich cells without cross-reaction on L cells meant that heterosynkaryons could not be detected with certainty. It is not known if these heterokaryons are capable of prolonged growth.

5. HeLa-hen erythrocyte and HeLa-chick erythrocyte heterokaryons

(i) Disappearance of antigen

The experiments of Schneeberger and Harris (1966) showed that it was the erythrocyte ghost which fused with HeLa cells and the erythrocyte cell membrane became continuous with the HeLa cell surface. It is

probably this antigen, carried into heterokaryons at the time of fusion, which has been detected with antiserum to erythrocytes. It is surprising that enough antigen is carried by one hen erythrocyte to result in strong labelling over the whole surface of a HeLa cell which has about 10 to 20 times the surface area.

Partial membrane fusion could explain the presence of chick antigen on single HeLa cells soon after fusion. The cells might separate before the completion of fusion because of agitation during the warming period or for some other reason. The disappearance of this antigen may represent turnover of HeLa surface components under normal conditions. The reason why chick antigen disappears more quickly and completely from single HeLa cells than from heterokaryons is uncertain. It may simply be that there is more hen antigen introduced into a heterokaryon than is left on single HeLa cells. The mechanism by which membrane mixing occurs is also completely unknown. There could be persistence of hen protein and/or mucopolysaccharide at the HeLa cell surface or complete mixing of the actual bimolecular lipoprotein leaflets. It is not known if persistence of recognisable hen antigen depends on all the components of the hen erythrocyte membrane being present at the surface of heterokaryons.

The disappearance of hen species antigen would appear to indicate that no hen protein is being synthesised in these heterokaryons. There is the possibility that hen antigen could be produced in heterokaryons but because of the structure of the HeLa membrane or the absence of chick cytoplasm could not appear on the HeLa surface. Moreover,

there is the theoretical possibility that hen antigen entering a predominantly HeLa surface might not be able to adopt a form recognisable by anti-hen erythrocyte antibody. Such a possibility was suggested by the work of Beisson and Sonneborn (1965) on paramecium. They found that there was hereditary maintenance of cortical variations of the paramecium surface in the absence of relevant nuclear differences. The presence, orientation and location of new cortical structures were determined by the cortical environment at the time of cell development. The following quotation from their work could apply to the surface antigens of HeLa-hen erythrocyte heterokaryons or to any heterokaryon system in which one cell type provides only a small proportion of the total cell surface: "Newly formed molecules do not enter a vacuum but a structured cell, the molecules of which are an essential part of the determinism for locating, orienting and patterning new molecular formations.".

Antigen molecules at the cell surface immediately after fusion could retain their morphology because there is mixing of entire membranes, but subsequent addition from below might not be possible. This hypothesis is not in fact true for these heterokaryons since recent work utilising specific antisera and soluble antigen extracted from HeLa-chick erythrocyte heterokaryons labelled with radioactive amino acids suggests that no intracellular hen protein is being produced (Gladstone and Wagner, unpublished observations).

The failure of hen antigen to be synthesised would agree with other observations on these heterokaryons. As has been noted by Harris (1967),

structures which resemble nucleoli appear only during the last stages of hen nucleus enlargement and they are often not visible in maximally enlarged nuclei. Nucleoli may be essential for the production of hen ribosomes (discussed by Harris, 1968) and hen ribosomes are probably essential for the synthesis of hen protein. Recent biochemical studies have shown that there is only "polydisperse" non-ribosomal RNA in hen nuclei isolated from these heterokaryons while the HeLa components synthesise polydisperse 16 S RNA as well as ribosomal 28 S and 16 S RNA (Bramwell, unpublished observations).

In summary one could postulate that hen antigen detected on HeLa-hen erythrocyte heterokaryons is left on the surface during the process of cell fusion. This antigen could be lost due to normal turnover of the HeLa cell surface. Failure to synthesise new hen antigen may be due to the failure of hen nuclei to synthesise ribosomal RNA.

(ii) Gamma-irradiation of HeLa cells

The use of gamma-irradiated HeLa cells for fusion experiments might be felt to add a further insult to cells already damaged by virus and by the introduction of a foreign nucleus, and for most heterokaryons, cytoplasm. However, it has been known for some time that X-irradiated cells will attach to glass (Puck and Marcus, 1955, 1956) and continue to synthesise DNA, RNA and protein after a low dose of radiation (Dickson, Paul and Davidson, 1958). Whitmore, Till, Gwatkin, Simino-vitch and Graham (1958) found that L cell mitosis was permanently stopped after 5,000 r but that the average DNA, RNA and protein increased logarithmically for 6 days at $\frac{1}{3}$ to $\frac{1}{2}$ the normal rate. This radiation

dose is comparable to that used in fusion experiments. Sheek, Des Armier, Sagik and Magee (1960) found that HeLa cells continued to synthesise DNA and protein normally for 10 days after a radiation dose of 1,400 r. These studies and others (Tolmach and Marcus, 1960) show that there may be a very great increase in the size of tissue culture cells after irradiation. The cell membrane is widely extended and actively motile and cell membrane processes such as pinocytosis proceed normally (Montgomery, Karney, Reynolds and McClendon, 1964).

Since heterokaryons formed with gamma-irradiated HeLa cells were studied only for 3 or 4 days, and since DNA, RNA and protein synthesis continues in these cells and many remain stuck to glass, it would seem valid to use them for study. Certainly irradiation makes it easier to follow many heterokaryons for a few days. Although these cells do not divide, there is abortive nuclear mitosis in some HeLa cells and heterokaryons 2 or 3 days after radiation, but there is usually no difficulty differentiating HeLa cells containing micronuclei from heterokaryons. Since these cells increase greatly in size, new membrane must be synthesised and the results of antigen studies on HeLa-chick erythrocyte heterokaryons showed that antigen disappeared from the surface of gamma-irradiated HeLa cells although at a slower rate than from normal cells.

(iii) Cytolysis

The reason why anti-chick erythrocyte antibody does not result in heterokaryon lysis is not understood. One suggestion made earlier for the failure of anti-Ehrlich antibody to lyse some HeLa-Ehrlich

heterokaryons was that the spatial distribution of Ehrlich antigen might prevent complement fixation. Such an explanation may be true here although some complement was apparently fixed as shown by immune adherence. The experiments of Spooner and Sell (1966) and Sell and Spooner (1966) were discussed earlier in connection with the cytolysis of HeLa-Ehrlich heterokaryons. These workers found that complement fixed by IgM anti-Forssman antibody resulted in weak and ineffective lysis of dog kidney cells although the same serum was highly haemolytic. Moller (1961) used the fluorescent antibody technique to demonstrate antibody to non-H-2 antigens on the surface of mouse cells but this antiserum was incapable of producing cytolysis. The explanation for all these results is not clear at the moment. Cytolysis may be affected not only by the concentration of particular surface antigens but also by their conformation.

6. Cell membrane turnover

Cell membrane turnover is suggested by the results with HeLa-hen erythrocyte heterokaryons and therefore the subject will be considered here. It is an important concept on which there is little definite data and hence will be discussed in some detail.

(i) Evidence from fusion of HeLa cells and erythrocytes

The disappearance of hen antigen from heterokaryons and single HeLa cells after cell fusion suggests that renewal of antigens at the surface of dividing HeLa cells is a process which takes 24 to 48 hours. Loss of chick antigen from gamma-irradiated HeLa cells does occur and suggests

that there is antigen turnover and replacement in non-multiplying cells.

The rate of antigen loss from heterokaryons was sometimes associated with the degree of chick nucleus enlargement. There was less chick antigen on heterokaryons with large chick nuclei. Harris (1967) showed that the degree of chick nucleus enlargement was proportional to the metabolic activity of HeLa cells. The most metabolically active HeLa cells were therefore losing chick antigen and this suggests active replacement of chick antigen rather than passive diffusion and loss. The problems of trying to answer this question with puromycin and actinomycin were illustrated in Results. Drug concentrations which did not affect autoradiographic labelling for RNA and protein synthesis caused HeLa cells to come off the glass.

(ii) Protein turnover

Turnover of protein and other substances in animal tissues was shown by the experiments of Schoenheimer (1942). Some studies on multiplying bacteria suggested that there was no protein turnover (Hogness, Cohn and Monod, 1955; Rotman and Spiegelman, 1954; Koch and Levy, 1955) but this has since been demonstrated in bacteria by Mandelstam (1957). One of the most convincing studies with animal cells in vitro was that of Harris and Watts (1958) who showed considerable protein turnover in rabbit macrophages. In cultured mammalian cells with a growth rate of about 3% an hour, an overall protein turnover of about 1% per hour has been suggested (Eagle, Piez and Fleischman, 1957; Eagle, Piez, Fleischman and Oyama, 1959;

Eagle and Levintow, 1965). Some proteins may be degraded and re-synthesised much more rapidly than others.

(iii) Theoretical evidence for membrane turnover

The possibility that there is turnover of the plasma membrane was suggested by the 'membrane flow' concept of Bennett (1956) and such an idea is favoured by Fawcett (1962) although the rate of membrane turnover is poorly understood. The processes of pinocytosis, phagocytosis, and glandular secretion would appear to indicate that cell membrane turnover must occur. Pinocytosis was first described by Lewis (1931) and the electron microscopic studies of Palade (1953) on capillary endothelium showed that pinocytosis vesicles were formed by invagination of the cell membrane. Fawcett (1962) feels that there are so many of these vesicles that the cell membrane would soon be used up if there were not local membrane synthesis or uniform addition of phospholipid and protein components from the cytoplasm molecule by molecule. Holtzer and Holtzer (1958-60) suggested that pinocytosis in macrophages implied synthesis of $\frac{1}{2}$ a new membrane in less than 4 hours.

Similar reasoning applies to the process of phagocytosis and there is a substantial increase in metabolism and turnover of phosphatides in phagocytosing leucocytes (Karnovsky and Wallach, 1961). Before the secretion of pancreatic enzymes zymogen granules acquire a smooth membrane in the Golgi area and during secretion of the enzymes this membrane becomes continuous with the cell plasma membrane (Fawcett, 1961, 1962). Continuous addition to the cell membrane implies that there must be equal withdrawal and hence circulation of the cell membrane.

(iv) Dynamic state of membrane lipids

Very little is known about protein turnover in cell membranes, but membrane lipids are in a dynamic state, at least in the erythrocyte. Altman, Watman and Solomon (1951) showed that acetate was rapidly incorporated into the stroma of the erythrocyte and that there was rapid turnover of C^{14}-labelled constituents of the stroma. This was contrasted with the metabolic stability of haemoglobin. Cholesterol can exchange between plasma and membrane lipoproteins (Hagerman and Gould, 1951; London and Schwarz, 1953). Some lipids can be washed out of the erythrocyte membrane (Lovelock, 1955). Erythrocytes can incorporate fatty acids into phospholipids (James, Lovelock and Webb, 1959; Mulder and van Deenen, 1965a) and exchange membrane and plasma phospholipids (Reed, 1959; Mulder and van Deenen, 1965b). This turnover of erythrocyte lipids must be contrasted with the stability of the lipids of myelin (Davison and Dobbing, 1960).

(v) Turnover of the cell membrane of non-vertebrate cells

Experiments with non-vertebrate cells cast some light on the process of membrane synthesis and turnover but must be applied to mammalian cells with caution. It was suggested by Selman and Waddington (1955) and Buck and Krishan (1965) that membrane synthesis for amphibian eggs occurs in the plane of cleavage at the time of cell division. New surface of sea urchin eggs may appear in the furrow region after cell cleavage (Wolpert, 1963) but the situation is complicated since the surface membrane is convoluted and surface area could increase at least 30% without new membrane being formed during cell cleavage.

Fluorescent antibody studies with yeast (May, 1962) have shown that new membrane is formed at the end of these cells. Similar studies by Cole and Hahn (1962) suggested that new cell wall of Streptococcus pyogenes appeared at the site of cross-wall formation and was not diffusely mixed with the old. However, later work with Salmonella (Cole, 1964) and E. coli (Beachey and Cole, 1966) suggested that there was diffuse insertion of new wall into old.

Fluorescent antibody studies with living amoebae (Wolpert and O'Neill, 1962) have shown that the label gradually accumulates in intracellular vacuoles and is lost from the cell surface in 12 hours. The surface was also noted to behave as a fluid envelope. It was felt that the surface coat and cell membrane moved as a whole in pinocytosis and phagocytosis but the mechanism of membrane replacement was completely unknown. Marshall and Nachmias (1965) suggested that actively feeding amoebae could consume an area of membrane equal to the entire cell surface in 5 minutes, while amoebae feeding steadily took in about 70% of the cell surface per hour. Basal membrane uptake by starved cells was about 1% per hour. Nachmias (1966) showed that when amoebae were coated with alcian blue the surface was replaced in 35 minutes. The process was much slower if cells were coated a second time.

These studies show that replacement of the cell membrane is usually a diffuse process but that the rate of replacement may vary widely with the method of study and with the cell type.

(vi) Synthesis of the cell membrane of vertebrate cells

The synthesis of cell membranes of vertebrate and mammalian cells is not well understood. According to Cook, Laico and Eylar (1965) the carbohydrate portion of the Ehrlich ascites cell membrane is added to the completed polypeptide chains in the membrane complex of the rough endoplasmic reticulum. They suggested that a sizeable pool of membrane existed in these cells and felt that the carbohydrate component turned over relatively slowly. As mentioned earlier a pool of membrane material could be one reason why Ehrlich antigen seems to persist on heterokaryons in the absence of RNA synthesis by the Ehrlich nucleus. Dallner, Siekevitz and Palade (1966) studied the membranes of the rough endoplasmic reticulum and suggested that the protein components of membranes are synthesised at the ribosomes. Lipids may be synthesised elsewhere but interaction with proteins seems to occur in the rough endoplasmic reticulum. Some studies implicate the Golgi region in the synthesis of cell membranes (Fawcett, 1961; Hicks, 1966) and material which stains like cell surface mucopolysaccharide has been demonstrated in this region (Gasic, Loebel and Badinez, 1960).

(vii) Regeneration of the cell surface of vertebrate cells

Although there have been no convincing studies on cell membrane turnover of vertebrate cells, there is some evidence that regeneration of surface components can occur. The work of Moscona suggesting that cell surface materials must be synthesised for the aggregation of embryonic cells has already been discussed. Gasic and Gasic (1962) demonstrated the mucopolysaccharide coat on mouse ascitic mammary

carcinoma cells by the Hale reaction. This layer could be removed with various enzymes and began to reappear after 1 or 2 hours. However, the cells were incubated in foetal calf serum - containing medium and the possibility that surface coatings were acquired from the serum does not appear to have been considered. Marcus and Hirsh (1963) removed HeLa cell surface receptors for Newcastle disease virus with neuraminidase and found that virus binding ability was restored within 20 hours. Similar results were reported by Kohn (1965).

Weiss and Kapes (1966) suggested that regeneration of cell surface materials necessary for cell adherence to glass occurred over a period of hours after enzyme treatment of the cells. Kraemer (1966) removed sialic acid from the surface of Chinese hamster cells with neuraminidase and found that control values were restored in 12 to 16 hours. If cell division was blocked with thymidine or actinomycin D, regeneration occurred at the same rate. Puromycin prevented regeneration of sialic acid but the massive dose used (50 μg/ml.) makes the results open to question. Kraemer felt that the slowness of restoration of sialic acid ruled out absorption from the medium but this possibility was not excluded. From this work he was uncertain if sialic acid terminal groups alone, larger molecules, or entire membrane segments had been replaced. The fact that the control amount of sialic acid was retained during prolonged treatment with puromycin was felt to indicate little or no turnover of cell surface sialic acid. However, it is probable that the large dose of puromycin killed the cells or perhaps prevented the synthetic and replacement processes which may be essential for

turnover to occur. Later work with glucosamine-1-^{14}C (Kraemer, 1967) has suggested that some turnover of sialic acid can occur. Thirty-six hours after exposure of cells to the radioactive precursor, surface sialic acid contained 55% and trypsin-removable material 36% of the initial radioactivity. However, these studies do not necessarily indicate turnover of surface antigens since the degree of antigen labelling as shown by mixed haemadsorption is unaffected by neuraminidase or trypsin treatment of tissue culture cells.

(viii) Conclusions

There is evidence for turnover of the surface of non-vertebrate cells and erythrocyte membrane lipids. Intracellular protein turnover occurs in mammalian cells and some surface components can regenerate. The study of HeLa-hen erythrocyte heterokaryons seems to indicate active replacement of the cell surface of dividing and non-dividing HeLa cells.

7. HeLa-chick fibroblast heterokaryons

The results of these experiments must be interpreted with some caution because of the difficulty in obtaining good mixed haemadsorption on single fibroblasts. The reason why chick antigen seems to persist on these heterokaryons but disappears from HeLa-chick erythrocyte heterokaryons is uncertain. The explanation could lie in the differences between the two cell surfaces, differences in the amount of chick antigen introduced at the time of fusion, or differences in the metabolic activity of each type of nucleus. Whole fibroblasts fuse with HeLa cells and so the heterokaryon contains chick cytoplasm which may

be necessary for antigen persistence or could act as a store of chick antigen. These heterokaryons are much more resistant to anti-chick antiserum than single fibroblasts. This provides a possible method of clearing heterokaryon cultures of single fibroblasts which otherwise could overgrow a culture in a few days.

8. HeLa-rat lymphocyte heterokaryons

There was a daily decrease in the amount of lymphocyte antigen on the surface of these heterokaryons and so the presence of some lymphocyte cytoplasm is not enough to ensure antigen persistence. Like erythrocyte nuclei in heterokaryons with HeLa cells, lymphocyte nuclei develop small nucleoli a few days after cell fusion, if nucleoli appear at all. Therefore ribosomal RNA may not be produced by the lymphocyte nuclei with the result that lymphocyte antigen cannot be synthesised.

Survival curves for HeLa-lymphocyte heterokaryons show that the presence of a lymphocyte nucleus in a HeLa cell and the mixture of lymphocyte and HeLa cell surfaces has no more effect on heterokaryon viability than the presence of an erythrocyte nucleus and surface. This suggests that it is not just contact of the lymphocyte surface with foreign antigens which leads to cell destruction but rather that some metabolic activity of lymphocytes may be necessary after contact with foreign antigens. Of course one must admit that the presence of a lymphocyte in a heterokaryon with a HeLa cell is a highly unnatural situation and may bear no relation to normal cell activities. In addition it is probable that a large proportion of lymphocytes are

incapable of cell destruction (Wilson, 1965). If only a few lymphocytes did cause heterokaryon death, this would not be detectable. An experiment of interest would be the fusion of lymphocytes with cells to which the lymphocyte donor has been sensitised. Would heterokaryon viability be affected by the presence of a specifically sensitised lymphocyte?

9. HeLa- and Ehrlich-macrophage heterokaryons

The reason for the disappearance of the macrophage property of adsorbing sensitised erythrocytes is not understood and the receptors themselves are not fully characterised. They could be covered by Ehrlich or HeLa surface antigens or lost during normal membrane turnover without the replacement processes being possible in heterokaryons. The latter may be the correct explanation, since the macrophage surface characteristic of sticking to glass and adsorbing sensitised erythrocytes is present 24 hours after the fusion of mouse macrophages and Ehrlich cells, but subsequently disappears. At least within the first 24 hours after fusion these receptors are not covered by Ehrlich surface material.

The fact that these heterokaryons retain the macrophage ability to stick to glass after the ability to adsorb sensitised erythrocytes is lost suggests that different surface receptors are involved in each function. The reason why Ehrlich macrophage heterokaryons die within a few days of cell fusion is unknown. Mouse macrophages are unusual cells and those from immunised mice are capable of specific cell destruction in tissue culture (Granger and Weiser, 1966).

The reaction of leucocidin with single macrophages and polymorphonuclear leucocytes is still uncertain but it may react with triphosphoinositide at the cell surface (Woodin, 1967). The failure of HeLa-rabbit macrophage heterokaryons to react with leucocidin may indicate that macrophage lipid receptors have been covered by the HeLa surface antigens, that the receptors have been lost during membrane turnover, or that the process of fusion and mixing of cell surfaces has caused a change in the configuration of the receptors so that the effect of leucocidin cannot be exerted.

10. Hamster fibroblast-L cell heterokaryons

No prolonged growth of these heterokaryons was detected but because double labelling was not used, no generalisations can be made. A subline of L cells has been used to produce a hybrid line with rat embryo cells (Weiss and Ephrussi, 1966a) and human embryo cells (Weiss and Green, 1967) so in theory hamster fibroblast-L cell heterokaryons should be capable of continued growth. The above studies did not use viral fusion but they did employ selective medium to prevent culture overgrowth by single cells and this may be very important. It is probable that hybrid cells could be produced and isolated by combining viral fusion with the selective techniques outlined by Littlefield (1964) and Davidson and Ephrussi (1965).

11. Transfer of antigen

The experiments in which L cells were grown on a HeLa feeder layer

show that antigen can be lost by gamma-irradiated HeLa cells and detected on cells of another strain. However, this problem was not serious since it was observed only for the few days during which irradiated cells were present in far greater numbers than growing cells. Some HeLa antigen did remain adherent to the coverslip after all HeLa cells had come off but the areas of mixed haemadsorption occurred between and not on the surface of growing L cells. These results could not explain labelling for HeLa antigen when hybrid cells were grown on a HeLa feeder layer.

Antigen has been detected in culture medium after growth of spleen cells in vitro by Mannick, Graziani and Egdahl (1964) and it was suggested that this poorly soluble material was released by cells undergoing growth and extensive lysis. The release of soluble H-2 antigens by hypotonic lysis of cells by Davies and his co-workers has already been discussed. It is possible that surface antigen loss, for example, of chick antigen from heterokaryons could occur in this way rather than by removal and intracellular breakdown of the membrane components. This would seem unlikely since most heterokaryons, at least within the first few days of fusion, are metabolically active and morphologically healthy, but such antigen disappearance could occur from dying heterokaryons some days after fusion.

The fact that antigen can be left on the glass after removal of tissue culture cells has been observed by other workers. Marcus (1962) found that areas of haemadsorption remained about the cell site after removal of myxovirus-infected HeLa cells with EDTA and trypsin. Weiss

and Lachmann (1964) showed that patches of antigenic material occurred about HeLa cells and it was suggested that this was due to microruptures of cytoplasmic processes. There was no diffuse zone of antigen encircling the cells. Weiss and Coombs (1963) found that cell surface antigen was left on glass after distraction of cells by agitation of the medium but this material could be removed by trypsin. These results suggest that the presence of a widely-spread antigenic zone about tissue culture cells may be particularly characteristic of pathological cells since this has been seen about virus-infected cells by Marcus and in my studies about gamma-irradiated HeLa cells and some heterokaryons.

12. Antibody persistence on HeLa cells

Studies on the persistence of antibody on the surface of tissue culture cells are theoretically very useful and might be compared with the disappearance of fluorescent antibody from amoebae (Wolpert and O'Neill, 1962) and bacteria (Beachey and Cole, 1966). Mammalian cells in tissue culture are, however, very sensitive to the toxic effect of the high concentrations of antibody necessary for fluorescence. It was essential to label cells with a low concentration of antibody and to have a sensitive technique for antibody detection. Mixed haemadsorption fulfilled this requirement.

Disappearance of hen antigen from HeLa-hen erythrocyte heterokaryons suggested that mammalian surface antigens turned over relatively slowly since hen antigen on single HeLa cells was not lost until 48 hours after fusion. Many things could happen to antibody on the cell surface

during this time. It could undergo degradation although control studies with dilute antibody in solution suggested that this did not occur. The antibody could elute to new or unlabelled antigen on the same or adjacent cells although this possibility was decreased by labelling cells with guinea pig antiserum and incubating the cells in dilute rabbit antiserum. Some antigen sites must remain unlabelled by the antibody concentrations used because higher concentrations of antiserum will kill cells even in the absence of complement.

It is possible that antibody on the cell surface might prevent normal antigen replacement in some way, perhaps by cross-linking adjacent antigens. Metz and Thompson (1967) have described a similar effect in which multivalent antibody prevented fertilisation and division of sea urchin eggs but univalent antibody did not. It was suggested that the effect of antibody was to cross-link neighbouring antigens. If this does occur with dilute antibody on tissue culture cells, it is an effect which does not alter cell multiplication or adherence.

Studies on the turnover of surface components measured in this way give only a minimum period for antigen persistence. If it can be shown that cells still have antibody on their surface at a certain time, that they are growing normally, and that there has been no antibody transfer from cell to cell or site to site on the same cell, then one can assume that antigen has persisted during this time. On the other hand, when antibody disappears one cannot say whether this represents antibody elution or normal turnover and replacement of the antigen. In addition surface antibody might in some way be recognised as foreign,

stimulate pinocytosis, and hasten the replacement process.

There are some ambiguities in this method, but these studies would appear to agree with the disappearance of hen antigen after cell fusion and suggest that replacement of surface antigens on growing tissue culture cells takes at least 2 days.

13. Conclusions

Information was obtained on each of the three objectives stated in the Introduction.

(i) The cell surface

The surfaces of different cells are capable of rapid and apparently even mixing. This emphasises the fluid and dynamic nature of the cell surface. Mixing of membranes with different properties and functions does not result in cell death. Cell membranes are apparently capable of some turnover as shown by the disappearance of hen antigen from heterokaryons.

(ii) Antigen labelling of heterokaryons

Mixed haemadsorption was shown to be a sensitive and effective method of labelling the antigens of heterokaryons and was applicable to living monolayer cells. Double labelling for different antigens with erythrocytes and bacteria was shown to be possible on the same heterokaryon. Mixed haemadsorption was very effective for screening cell populations for division and was used to demonstrate HeLa-Ehrlich hybrid clones. Mixed haemadsorption could also be used for cloning but no prolonged hybrid division was detected.

(iii) Protein synthesis

The loss of hen antigen from heterokaryons suggested that no hen protein was synthesised in heterokaryons in spite of RNA synthesis by hen nuclei. The persistence of Ehrlich antigen on HeLa-Ehrlich heterokaryons in spite of the use of UV-irradiation to prevent RNA synthesis by Ehrlich nuclei is not understood. Ehrlich antigen may remain from the time of fusion due to the amount of antigen introduced, due to the impaired metabolism of heterokaryons which do not live more than a few weeks, or to continued antigen synthesis in the absence of RNA synthesis.

These and other observations such as the loss of specialised cell surface characteristics and the unusual morphology and movement of heterokaryons may not be explained with certainty until many aspects of cell surface structure and function are better understood. For future studies it would be of value to follow the behaviour of particular blood-group and tumour-specific antigens. Work employing antiserum to whole cells and mixed haemadsorption is subject to some uncertainty because of the wide range of antigens being detected.

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ABSTRACT

A study has been carried out on the surface antigens and surface properties of heterokaryons formed by fusing animal cells of different species with UV-inactivated Sendai virus. HeLa cells of human origin were fused with mouse Ehrlich ascites cells, mouse L strain fibroblasts, hen and chick erythrocytes, rat lymphocytes, chick fibroblasts, and mouse and rabbit macrophages. L cells were fused with Ehrlich cells and with hamster embryo fibroblasts.

In the first section of the thesis experiments using inactivated virus to produce artificial heterokaryons were reviewed. The structure and function of the cell membrane and cell surface were considered as well as some of the methods, problems and results of antigen identification in tissue culture.

In experimental work the mixed antiglobulin technique of mixed haemadsorption was found to combine great sensitivity with effective labelling and was applicable to living monolayer cells. It was the main method used for studying the surface antigens of heterokaryons.

HeLa-Ehrlich heterokaryons and mononuclear hybrid cells carried both human and mouse surface antigens and these appeared to be intimately mixed. A double labelling technique using erythrocytes and killed bacteria as marker particles was developed and permitted each species antigen to be identified on the same cell. Small clones of fibroblastic cells bearing each surface antigen appeared some days after fusion, but the division rate was slow and hybrid cells did not live

more than a few weeks.

HeLa-Ehrlich heterokaryons could be lysed with antiserum against each species antigen but were slightly more resistant to the cytolytic effect of anti-HeLa serum than single HeLa cells and considerably more resistant to anti-Ehrlich serum than single Ehrlich cells. Combinations of dilute antisera were more effective in producing cytolysis than single antisera at the same concentrations.

Heterokaryons were also produced by the fusion of HeLa cells with UV-irradiated Ehrlich cells. A combination of autoradiography and mixed haemadsorption showed that Ehrlich antigen could persist on heterokaryons in the absence of RNA synthesis by Ehrlich nuclei.

HeLa-L cell heterokaryons were also found to carry the antigens of each species but only occasional pairs of mononuclear hybrid cells were seen and no hybrid clones.

Hen species antigen was shown on HeLa-hen erythrocyte and HeLa-chick erythrocyte heterokaryons, but the percentage of heterokaryons carrying this antigen decreased daily after cell fusion in spite of RNA synthesis by enlarging hen or chick nuclei. The presence of hen antigen on some single HeLa cells soon after fusion was thought to represent incomplete fusion of erythrocytes. The disappearance of this antigen within 24 or 48 hours could indicate turnover and synthesis of HeLa cell surface. The theoretical and experimental evidence for cell membrane turnover was reviewed and the lack of definite data noted.

HeLa-chick fibroblast heterokaryons could be identified for three

days and the proportion labelled for chick antigen did not decrease.

Rat species antigen was demonstrated on HeLa-rat lymphocyte heterokaryons but disappeared daily following fusion in a pattern similar to the loss of chick antigen in the HeLa-erythrocyte system. Gamma-irradiation of HeLa cells prior to cell fusion was found to delay nuclear fusion and prolong the life-span of HeLa-lymphocyte and HeLa-erythrocyte heterokaryons.

Ehrlich-mouse macrophage heterokaryons adhered to glass. The macrophage surface property of adsorbing erythrocytes sensitised with antibody was expressed on these heterokaryons initially but lost within two days of fusion. HeLa-rabbit macrophage heterokaryons lost the ability to adsorb sensitised erythrocytes within 24 hours of fusion and were not affected by concentrations of leucocidin which killed all single macrophages.

A series of experiments was performed in which HeLa cells were labelled with dilute antibody which did not affect cell multiplication. A method of preventing antibody transfer was developed and antibody labelled cells were detected daily by mixed haemadsorption. The possible use of such a method in studying antigen turnover was considered.

Heterokaryon surface characteristics such as persistence of parental antigens on some heterokaryons and disappearance of antigens from others, loss of specialised surface properties, and unusual morphology and surface activity were discussed. A correct explanation may not be possible until the structure, synthesis and metabolism of the cell surface are better understood.