The regulated long-term delivery of therapeutic proteins by using antigen-specific B lymphocytes

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Memory lymphocytes are important mediators of the immune response. These cells are long-lived and undergo clonal expansion upon reexposure to specific antigen, differentiating into effector cells that secrete Ig or cytokines while maintaining a residual pool of memory T and B lymphocytes. Here, the ability of antigen-specific lymphocytes to undergo repeated cycles of antigen-driven clonal expansion and contraction is exploited in a therapeutic protocol aimed at regulating protein delivery. The principle of this strategy is to introduce genes encoding proteins of therapeutic interest into a small number of antigen-specific B lymphocytes. Output of therapeutic protein can then be regulated *in vivo* by manipulating the size of the responder population by antigen challenge. To evaluate whether such an approach is feasible, we developed a mouse model system in which Eμ- and Igλ-based vectors were used to express human erythropoietin (h*EPO*) gene in B lymphocytes. These mice were then immunized with the model antigen phycoerythrin (PE), and immune splenocytes (or purified PE-specific B lymphocytes) were adoptively transferred to normal or mutant (EPO-deficient) hosts. High levels of hEPO were detected in the serum of adoptively transferred normal mice after PE administration, and this responsiveness was maintained for several months. Similarly, in EPO-deficient anemic recipients, antigen-driven hEPO expression was shown to restore hematocrit levels to normal. These results show that antigen-mediated regulation of memory lymphocytes can be used as a strategy for delivering therapeutic proteins *in vivo*.

Conventional therapies for inherited or acquired serum protein deficiencies generally require the systemic administration of appropriate exogenous proteins. In most cases, a continuous supply of the recombinant or surrogate protein is required, and patients are therefore subjected to repeated administration of the protein for extensive periods. In principle, somatic gene therapy offers the possibility of *in situ* production of the missing or dysfunctional products, thereby eliminating the dependence on frequent injections of exogenous proteins. This therapy may be aimed either at correcting the intrinsic defect in a specific cell type or by directing transduced cells to secrete the protein of interest into the bloodstream, making it available to other tissues. In practice, both approaches have been difficult to apply successfully, and the dual requirements of sustained long-term correction and regulated expression of therapeutic factors remain a major challenge.

Over the past decade, gene therapy protocols using various host cells and different types of vectors to regulate transgene expression have been used to engineer therapeutic options for several disorders with known genetic etiology (1–8). In the relatively near future, it is likely that the genetic basis of many more human diseases will be discovered, once a detailed knowledge of the human genome has been extended. With this increasing knowledge, there will be an opportunity to devise corrective strategies. Here, we investigate whether antigen-specific lymphocytes can be used as cellular vehicles for delivering therapeutic proteins in an antigen-regulated and systemic fashion for prolonged periods of time. These cells offer some advantageous features, such as longevity (9), high rate secretion of proteins (Ig or cytokines) (10, 11), and the potential to recognize and respond to specific antigen by proliferation, secretion, and memory cell formation (12, 13). We reasoned that if antigen-specific memory lymphocytes could be harnessed to express a gene of therapeutic interest, then repeated cycles of antigen-driven clonal expansion and contraction could be used to regulate the size of the responding transgene-expressing cell population (and thereby transgene output) according to antigenic challenge. Because lymphocytes have direct access to the bloodstream, they also are ideally placed for systemic distribution of transgene-encoded protein. These attributes, when used in conjunction with an increasingly sophisticated range of gene regulatory elements (14, 15), allowed us to develop a mouse model system to test the efficacy of antigen-specific B lymphocytes for protein delivery. Here, we show that antigen-specific B lymphocytes transduced with a model therapeutic gene, human erythropoietin (hEPO), survive for several months upon transfer into normal recipients and secrete hEPO specifically in response to antigen challenge. We demonstrate that this approach leads to elevated hEPO levels in normal recipient mice but, more importantly, can be used for a long-term correction of anemia in EPO-deficient animals.

Methods

Generation and Characterization of λ1-hEPO Transgenic Mice. The Eμ and λ1 cassettes containing hEPO-coding sequences were generated as follows. A 1-kb *Xba*I fragment of the pSV-Vmu1 plasmid containing the Eμ enhancer (16) was cloned into the *Sac*I site of Bluescript II SK (+/−) (Stratagene). The promoter, a 562-bp upstream fragment (−630 to −68 relative to ATG) of the rearranged mouse Igλ light chain, was PCR-amplified from clone pλ7.2 (A.M. and N.D., unpublished data) and inserted between the *Sac*II and *Not*I sites of Bluescript. A 2.5-kb *Sal*I/*Xho*I fragment at the 3′ region of the human β-globin gene [from exon 2 to beyond the polyadenylation site and the "upstream mouse sequence" (UMS) (17)] was cloned into the *Sal*I and *Xho*I sites. In the λ1 cassette, Eμ was replaced by λ1-3′HS1-4, a 2.9-kb fragment [including the $E\lambda_{3\text{-}1}$ enhancer (18) and three additional DNase I hypersensitive sites; A.M. and N.D., unpublished data] that was inserted into the *Xho*I site of Bluescript downstream of the UMS. hEPO was generated by PCR amplification of genomic DNA to yield a 2.5-kb fragment comprising the entire gene (from −260 bp upstream to ATG to 50 bp downstream to TGA) and inserted between the *Not*I and *Hin*dIII sites in Bluescript.

Purified DNA containing a *Bss*HII fragment of λ1-hEPO was

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Abbreviations: EPO, erythropoietin; hEPO, human EPO; LPS, lipopolysaccharide; PE, phycoerythrin; UMS, upstream mouse sequence.

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microinjected into fertilized eggs from (C57BL/6 × CBA)F_1 mice, and transgenic animals were identified by using PCR. The number of integrated transgene copies in progeny derived from individual founder mice was analyzed by Southern blotting. For Northern blot analysis, total RNA from transgenic and control mice was isolated by the RNAzol B method (Biogenesis). RNA (10 μg) was fractionated by formaldehyde-containing gel, blotted on nylon membrane (Hybond, Amersham Pharmacia), and hybridized with ^{32}P-labeled 2.5-kb hEPO genomic DNA probe. The membranes were washed at high stringency.

Lymphocyte Stimulation and Detection of hEPO Protein by ELISA. Splenocytes, derived from hEPO transgenic or control mice, were cultured with polyclonal B cell lipopolysaccharide (LPS) (25 μg/ml) under standard tissue culture conditions, and supernatants were harvested 48 h later.

hEPO was detected by using a commercial hEPO ELISA kit (Roche Diagnostics) that did not detect endogenous mouse EPO in normal sera (<2.8 milliunits/ml). Occasionally, after larger-volume (250 μl) bleeding, we experienced some cross-reactivity, detecting EPO (<10 milliunits/ml) in nontransgenic animals. To avoid the stimulation of endogenous EPO secretion, we minimized the volume of blood samples (50–100 μl) taken from the tail veins.

Hematocrit values were determined by standard centrifugation.

Cell Transfer Experiments. ***Mice and immunizations.*** hEPO transgenic cell donor mice (line 246) were back-crossed (at least six generations) to C57BL/6 mice. Six- to 8-week-old female mice that were heterozygous for the h*EPO* transgene were immunized intraperitoneally with 100 μg of alum-precipitated phycoerythrin (PE) and 10^9 heat-inactivated *Bordetella pertussis*. Two weeks after the priming unfractionated splenocytes (0.8–5.0×10^7), purified B cells (3.5×10^6–10^7), PE-positive/negative B cells (4×10^4), or equivalent wild-type cells were injected into the tail vein of PE-immunized, nonirradiated C57BL/6 female recipients. Where stated, we used (C57BL/6 × 134.3 LC)F_1 Epo-TAg heterozygous or C57BL/6 Epo-TAg homozygous mutant mice as recipients. Recipients were injected with soluble PE or PBS on the day of cell transfer and at different subsequent times. Blood samples (50–100 μl) were taken weekly to monitor serum hEPO concentrations and/or hematocrit values. All animal experimental procedures used in this study were performed in accordance with Home Office regulations.

Cell purifications. Splenic B cells from PE-immunized hEPO transgenic or nontransgenic C57BL/6 mice were purified by using a magnetic immunoselection with commercially provided reagents (Miltenyi Biotec, Auburn, CA). Briefly, single-cell suspensions of splenocytes were incubated with either anti-CD43 (Ly-48)-coupled microbeads or a combination of biotinylated anti-B220 antibody (Pharmingen) and streptavidin microbeads according to manufacturer instructions. The B lymphocytes were negatively (or positively) selected by passing the cells through an appropriate column. PE-specific B cells were isolated from this purified B cell population by fluorescence-activated cell sorter (FACS) analysis with FITC-conjugated anti-B220 antibody (Pharmingen) and PE.

Results

Expression and Biological Activity of Therapeutic Proteins in B Lymphocytes. To evaluate whether B lymphocytes can be used to express human proteins such as EPO, we established transgenic mice from which antigen-specific B cells could be readily isolated after immunization. This was done to facilitate the experimental transfer of defined populations of lymphocytes that express therapeutic genes of interest and to allow us to rigorously examine the tissue specificity and integration site-dependence of each construct. As shown in Fig. 1*a*, two different vector cassettes were generated into which the h*EPO* gene was inserted. The Eμ-based vector contains the IgH enhancer, the Igλ1 promoter, the human β-globin gene polyadenylation signal, and the UMS that was included to facilitate efficient transcriptional termination. In the λ1-based vector, the Eμ enhancer is replaced with the λ1–3′HS1-4 fragment. We inserted the h*EPO* gene as a model therapeutic gene into these cassettes and generated transgenic mice.

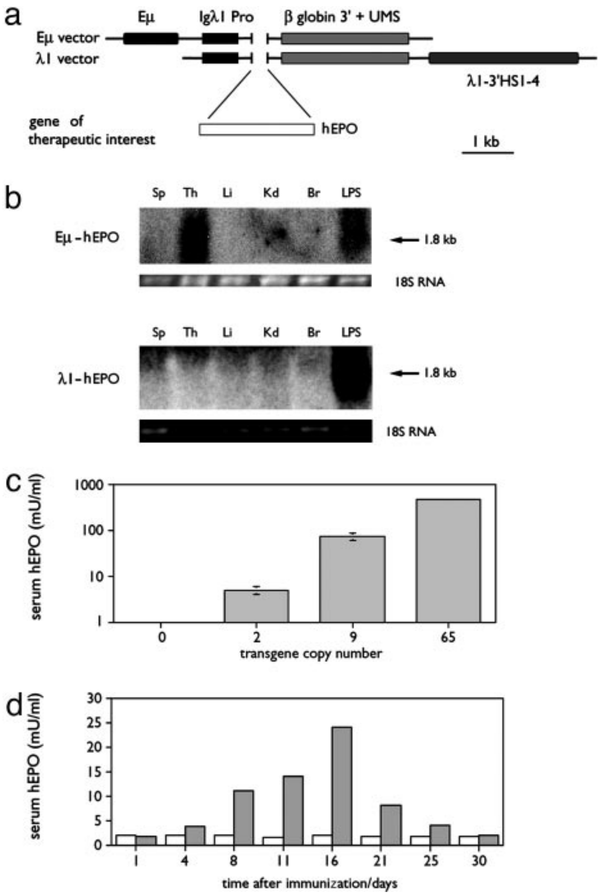

Fig. 1. Expression of therapeutic proteins by mouse lymphocytes. (*a*) The construction of Eμ- and λ1-based vectors used to generate transgenic mice, where Eμ indicates the Ig heavy chain enhancer, and Igλ1 Pro labels the Ig λ1 promoter. β-globin 3′+UMS denotes a fragment encompassing the 3′ end of human β-globin gene and transcription terminator UMS, followed by λ1–3′HS1-4, the 3′ Igλ1 enhancer. As a model therapeutic gene, h*EPO* was inserted into the Eμ- and λ1-based cassettes as shown. (*b*) Northern blots of total RNA extracted from different transgenic tissues; spleen (Sp), thymus (Th), liver (Li), kidney (Kd), brain (Br), as well as LPS-stimulated splenocytes. The arrows indicate the major 1.8-kb hEPO transgene-derived transcripts. 18S ribosomal RNA bands stained with ethidium bromide are shown to illustrate the equivalence of RNA loading between samples. (Transgene expression was confirmed in two independent Eμ-hEPO and λ1-hEPO lines, respectively.) (*c*) hEPO expression by polyclonally stimulated transgenic splenocytes. hEPO concentrations detected in the supernatants of 48 h *in vitro* cultures of 3×10^6 LPS-stimulated splenocytes isolated from independent hEPO transgenic lines with different numbers of integrated transgene copies are shown. Mice used in these experiments were age-matched, and the mean values obtained from two independent cell cultures are shown. (*d*) Antigen-induced hEPO production in hEPO transgenic mice *in vivo*. The shaded histogram profile illustrates hEPO levels detected in a transgenic mouse (line 339) immunized with PE on day 1. Serum hEPO levels of an untreated, hEPO transgenic littermate are shown for comparison (open histograms). A representative profile of three independent experiments is shown.

Transgene expression was initially analyzed by Northern blot analysis of RNA prepared from tissues isolated from individual

Table 1. Summary of λ1-hEPO transgenic lines, showing a correlation between transgene copy number, hEPO expression, and hematocrit

Transgenic lines	Transgene copy no.	Serum hEPO, milliunits/ml 6 weeks	Serum hEPO, milliunits/ml 12 weeks	Hematocrit % 6 weeks	Hematocrit % 12 weeks
260	2	3.0 ± 2.1	4.2 ± 1.7	48.1 ± 3.2 (6)	50 ± 1.0 (3)
339	2	3.7 ± 2.5	4.1 ± 2.1	47.0 ± 2.0 (3)	52 ± 1.5 (3)
246	4	54 ± 8.0	210 ± 6.0	67.6 ± 2.4 (9)	80 ± 3.0 (9)
341	9	82 ± 25	240 ± 82	66.8 ± 3.6 (6)	79 ± 3.2 (6)
305	65	361 ± 98	520 ± 75	79.4 ± 3.2 (7)	85 ± 2.5 (4)
Nontransgenic	—	<2.8	<2.8	42.0 ± 2.2 (18)	42.5 ± 2.5 (23)
Epo-TAg +/−	—	—	—	nd	38.1 ± 1.1 (28)
Epo-TAg −/−	—	—	—	nd	18.6 ± 1.7 (9)

Values are given as mean ± SD. The number of mice sampled for each determination is shown in brackets. Mice in which the endogenous EPO locus is disrupted (Epo-TAg) are anemic (22) and were used as recipients in some adoptive transfer experiments (Fig. 3). —, not applicable; nd, not determined.

progeny of Eμ-hEPO and λ1-hEPO lines, and representative examples are shown in Fig. 1*b Upper* and *Lower*. In Eμ-hEPO mice, transgene expression was abundant in RNA samples prepared from the thymus but not in liver, kidney, and brain samples. In primary splenocytes, transgene-derived RNA was poorly detected, but after stimulation with LPS, a marked increase in expression was observed (Fig. 1*b Upper*). In λ1-hEPO lines, transgene expression was detected exclusively in LPS-activated splenocytes (Fig. 1*b Lower*). These results show that Eμ- and λ1-based vectors drive transgene expression in different lymphocyte subsets, and that λ1-based vectors might be particularly useful for expressing candidate genes in activated mature B lymphocytes.

To examine this in more detail, five independent λ1-hEPO transgenic lines were derived and analyzed (Table 1). hEPO was routinely detected in the serum of founders and progeny from each of these transgenic lines, and it increased with age (compare 6 and 12 weeks). In addition, hEPO was readily detected in culture supernatants harvested from primary transgenic B cells after *in vitro* culture with LPS (Fig. 1*c*). Interestingly, we observed a strong correlation between the amount of hEPO detected *in vivo* (in the serum) or *in vitro* (in LPS-stimulated cultures) and transgene copy number (Table 1 and Fig. 1*c*). In addition, low-copy-number mice (lines 260 and 339) showed only a mild elevation in hematocrit values (48–52%), whereas high-copy-number mice (lines 341 and 305) had increased hematocrits (79–85%) (Table 1), enlarged spleens, and elevated red blood cell counts. These data demonstrate that lymphocyte-derived hEPO was biologically active *in vivo* and suggest that expression of the λ1-hEPO transgene was copy number-dependent and relatively independent of integration site.

Immunization of these mice with antigen also elicited hEPO protein production in the serum. Fig. 1*d* shows data from two λ1-hEPO transgenic littermates (line 339), one of which was immunized with PE at day 1. In this mouse, the concentration of hEPO increased from <2 milliunits of hEPO per ml of serum to a peak of 24 milliunits/ml 16 days after PE immunization (filled bars). Levels then gradually declined to those of the nonimmunized transgenic control (open bars). The kinetics of this response are largely consistent with the predicted time course of Ig production in a primary immune response (19). The slightly steeper decline in hEPO levels (compared with that predicted for Ig) most likely reflects the shorter *in vivo* half-life of erythropoietin (8 h) (20), as compared with Ig (1 week) (21).

Regulated Production of hEPO in Normal Mice After the Adoptive Transfer of Antigen-Specific hEPO Transgenic Lymphocytes. To evaluate the survival and responsiveness of antigen-specific hEPO-expressing splenocytes in normal (i.e., nontransgenic) recipients, a series of adoptive transfer experiments were performed in which donor-derived hEPO was monitored in the serum of recipients after transfer and antigen administration (Fig. 2).

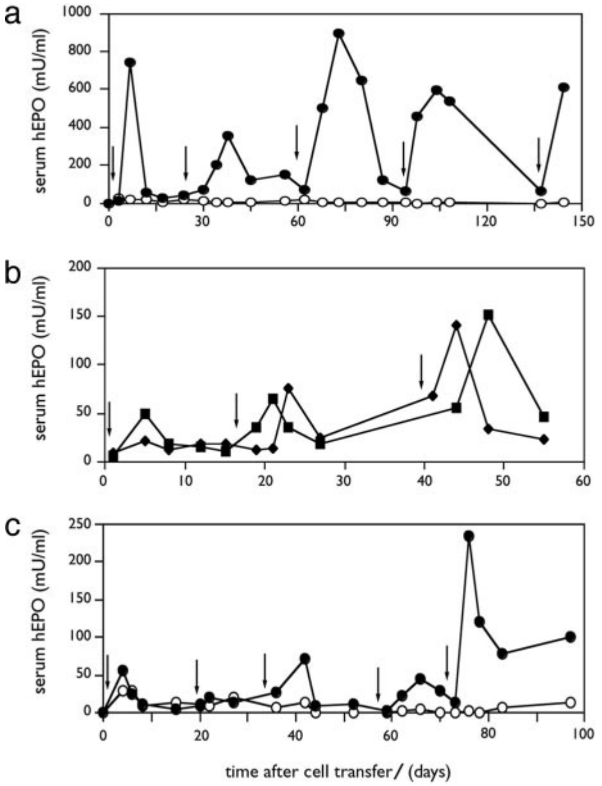

Fig. 2. Regulated production of hEPO in normal mice after the adoptive transfer of antigen-specific hEPO transgenic lymphocytes. (*a*) The different responses of mice transferred with 3 × 10^7 unfractionated, PE-primed λ1-hEPO splenocytes, challenged with either soluble PE (filled circles) or PBS (open circles) at different time points after cell transfer (indicated by arrows). Serum hEPO levels were monitored for 145 days. (*b*) 3 × 10^6 CD43-depleted lymphocytes purified from a PE-primed λ1-hEPO transgenic mouse were transferred into two normal recipients that were injected with soluble PE at different times after transfer (arrows), and the level of hEPO in their sera was monitored for 56 days. (*c*) Transfer of 4 × 10^4 PE-binding (filled circles) and PE-nonreactive (open circles) λ1-hEPO transgenic B cells into two normal recipients. Both mice were boosted with soluble PE at the indicated times (arrows), and serum hEPO levels were monitored for 98 days.

Initially, a single λ1-hEPO transgenic mouse (line 246, donor) was immunized twice with PE (days 0 and 14). One month after the first immunization, 3×10^7 unfractionated splenocytes were transferred intravenously into two PE-primed, nontransgenic recipients. One recipient also was injected with soluble PE (Fig. 2*a*, filled circles), whereas the other received PBS alone (open circles). By day 7 after cell transfer, serum hEPO levels had increased significantly in the PE-boosted animal (744 milliunits/ml) but not in the PBS-immunized recipient. Levels of hEPO declined between days 12 and 28 (30–50 milliunits/ml), but additional injections of soluble PE on days 28, 60, 92, and 137 induced peak responses of 356, 891, 599, and 613 milliunits/ml ≈10 days after each challenge. These experiments demonstrated that transgenic splenocytes secreted high levels of therapeutic protein in response to antigen challenge. Moreover, additional experiments (data not shown) suggested that the level of hEPO generated in these recipients depended on the number of transferred splenocytes.

To test whether transgene production also could be achieved by transferring purified B cells alone, parallel experiments were performed by using donor cell populations that were rigorously depleted of CD43-expressing cells (including T cells, granulocytes, and plasma cells). In these experiments, two normal recipients were each given 3.5×10^6 CD43-depleted splenocytes isolated from a PE-immunized transgenic donor (line 246). Recipients were immunized with soluble PE on the day of cell transfer and subsequently on days 19 and 40 (Fig. 2*b*, arrows). Peak serum hEPO levels were detected in mice a few days after each PE boost (≈50, 75, and 150 milliunits/ml, respectively).

To ensure that hEPO secretion was derived from the antigen-specific population of B cells among the transferred population of CD43-depleted cells, PE-reactive cells were purified and examined in adoptive transfer experiments into normal hosts. Here, as shown in Fig. 2*c*, both recipient mice received a small number (4×10^4) of CD43-depleted transgenic B lymphocytes that were selected for PE binding (PE-reactive, filled circles) or depleted of PE-binding cells (open circles). Injection of recipients with soluble PE at the time of cell transfer and four additional times (arrows) provoked an increase in serum hEPO (56–233 milliunits/ml) only in the mouse that received PE-reactive donor cells. This experiment confirmed the capacity of PE-specific B lymphocytes to deliver therapeutic protein in a specific, antigen-responsive manner over a prolonged period *in vivo*. Consistent with the delivery of biologically active hEPO, an increased hematocrit was measured at the termination of the experiment (day 187) in the recipient of PE-reactive B cells (53%) but not in the mouse that had been transferred with PE-depleted B cells (39%).

Correction of Anemia in EPO-Deficient Mice Can Be Achieved by Transferring λ1-hEPO Transgenic B Lymphocytes. The possibility that hEPO-secreting B lymphocytes could be used to ameliorate anemia in genetically mutant (EPO-deficient) mice was assessed by performing cell transfer experiments with 134.3LC Epo-TAg mice as recipients. These mice have a targeted disruption in the 5′ untranslated region of the *EPO* gene disabling its function (22). Homozygous mice in which both copies of the *EPO* gene are modified are severely anemic (hematocrit 18.6 ± 1.7%), whereas heterozygous mice showed moderate but significant anemia (hematocrit 38.1 ± 1.1%; Table 1), as reported in ref. 22.

We tested whether the adoptive transfer of hEPO-transduced antigen-specific B lymphocytes could restore hematocrit levels in hemizygous and homozygous mutant recipients. Heterozygous Epo-TAg transgenic mice that are chronically anemic (Table 1) were restored to health, with hematocrits in the normal range, after the transfer of 5×10^6 CD43-depleted, PE-primed λ1-hEPO (line 246) transgenic splenocytes (Fig. 3*a*, filled circles, $n = 4$) and PE immunization (as indicated by arrows). Parallel experiments in which an equivalent number of CD43-depleted splenocytes from PE-primed wild-type donors were transferred (Fig. 3*a*, open circles, $n = 4$) failed to correct the anemic profile of these mice over the 3-month observation period. The possibility that an inadvertent transfer of red-cell precursors in hEPO transgenic donors (that populate the spleen and might not be removed by CD43 depletion) could have contributed to the correction of anemia was addressed by directly purifying B220-positive cells. Approximately 10^7 B220-positive splenocytes purified from the PE-primed transgenic (Fig. 3*b*, filled circles) and normal donors (Fig. 3*b*, open circles) were transferred into heterozygous Epo-TAg mice. Consistent with previous results, transfer of hEPO transgenic B cells resulted in an elevation of hematocrit values in heterozygous Epo-TAg mice reaching 60% on day 17 and remaining around this value for at least 3 months. On the contrary, recipients of wild-type B cells showed no dramatic changes in hematocrit (35–42%).

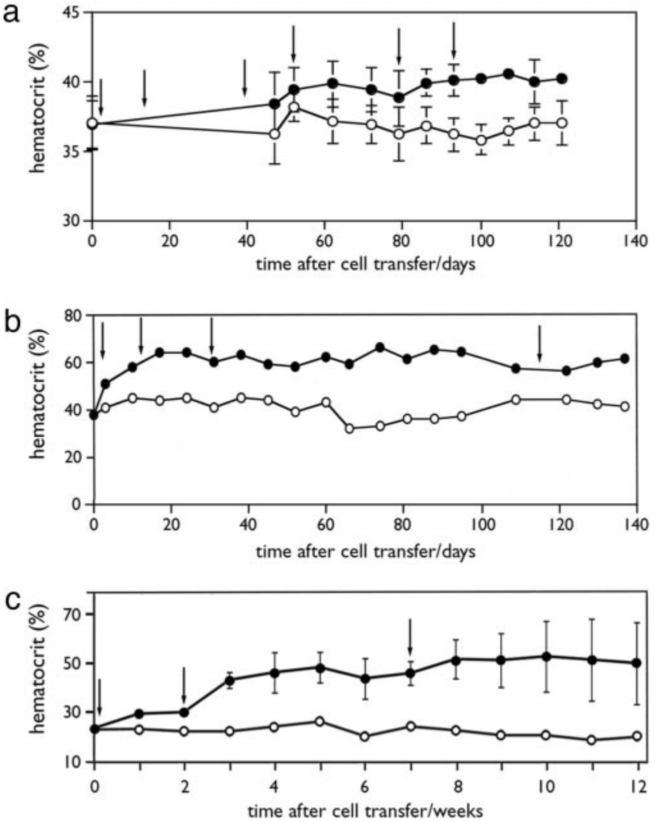

Fig. 3. Correcting anemia in mutant mice by transferring bone marrow cells or PE-specific B lymphocytes from λ1-hEPO transgenic donors. (*a*) 5×10^6 CD43-depleted B cells purified from PE-immunized hEPO transgenic (filled circles) or wild-type (open circles) donors were injected into two groups of four heterozygous Epo-TAg anemic mice. Both sets of mice were injected with PE on the day of cell transfer and additionally on days 16, 40, 51, 79, and 94 (arrows). Their hematocrit values were monitored between days 45 and 120. The data shown are the mean ± SD of hematocrits. (*b*) The differing responses of heterozygous Epo-TAg mice that received 10^7 purified B220-positive (filled circles) or B220-negative (open circles) PE-primed hEPO transgenic splenocytes. The hematocrit values and PE immunization schedule (arrows) are shown. (*c*) Five severely anemic homozygous Epo-TAg mice were adoptively transferred with 5×10^6 B220-positive λ1-hEPO transgenic B lymphocytes separated from PE-immunized donors. The recipients were injected with PE on the day of cell transfer, 2 and 7 weeks later (arrows). Mean hematocrit values of five mice and standard deviations are shown (filled circles). A control Epo-TAg mouse (open circles) was transferred with 5×10^6 B cells derived from PE-immunized wild-type donor and injected with PBS (arrows).

To further test the capacity of antigen-regulated hEPO trans-

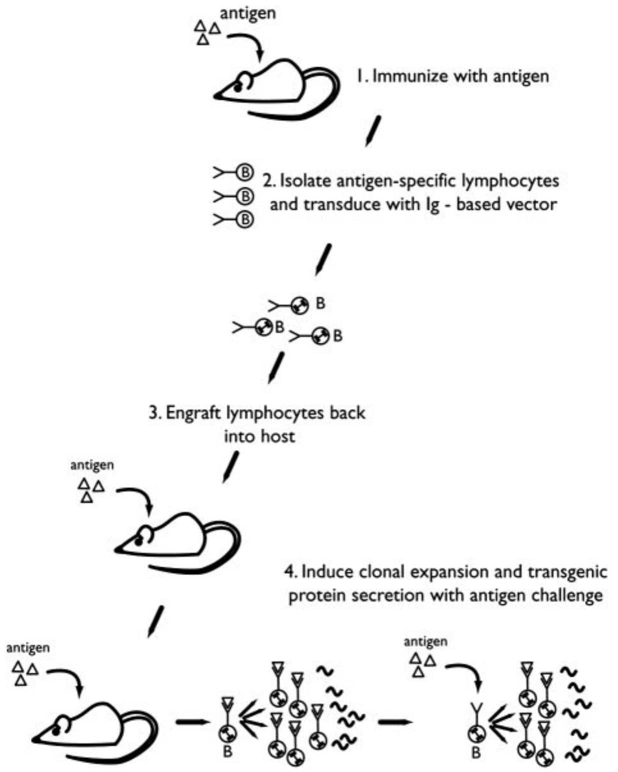

Fig. 4. Schematic representation of the immunoregulated gene therapy approach.

genic B lymphocytes to correct severe anemia, we performed similar adoptive transfer experiments on homozygous Epo-TAg mice. B220-positive splenocytes were separately isolated from PE-immunized λ1-hEPO transgenic and PE-immunized wild-type donors and transferred to homozygous Epo-TAg mice (Fig. 3*c*). Five recipients that received 5×10^6 hEPO transgenic B lymphocytes (and PE injections on the day of cell transfer, 2 and 7 weeks later) showed a marked increase in hematocrit reaching normal values (32–55%) by week 3 and remaining in this range over the next 12 weeks (Fig. 3*c*, filled circles). In contrast, a homozygous Epo-TAg control mouse receiving 5×10^6 B cells derived from wild-type PE-immunized donor (and PBS injections at the indicated times) did not show any increase in hematocrit and remained severely anemic throughout the experiment (Fig. 3*c*, open circles). These results show unequivocally that antigen-specific hEPO-secreting transgenic B cells can be used *in vivo* to correct severe anemia caused by an intrinsic genetic deficit in EPO production.

Prototype Strategy for Immunoregulated Gene Therapy. On the basis of our results, a four-part therapeutic protocol can be proposed as outlined in Fig. 4. In this protocol, the host is first immunized with antigen (for example, PE) and antigen-specific B lymphocytes are isolated a few weeks later from the blood or spleen. These cells are transduced with a gene of therapeutic interest (h*EPO* is exemplified in this study) under the control of appropriate lymphocyte-specific gene regulatory elements. Transgene-containing antigen-specific B cells are then reintroduced into the host and reexposed to antigen to undergo clonal expansion, transgene-coded protein secretion, and memory cell formation.

Discussion

Transfected lymphocytes have been used in different areas of clinical therapy to correct deficiencies (23–25), in experimental gene therapy protocols to induce tolerance (26, 27), or to treat autoimmune diseases (28–30). Although there is growing experience in using T cells for gene therapy purposes, B lymphocytes have received less attention (31). Here, we describe an approach for achieving long-term and regulated expression of therapeutic proteins *in vivo*. In a mouse model system, we show that antigen-specific B lymphocytes transferred from λ1-hEPO transgenic mice into normal recipients can be stimulated to secrete hEPO *in vivo* in an antigen-responsive manner for several months, and by using this approach, severe chronic anemia can be efficiently corrected. This study provides "proof of principle" that antigen-specific B lymphocytes can be applied as cellular vehicles for long-term, systemic, and antigen-regulated delivery of therapeutic proteins *in vivo*.

In this study, we developed transgenic mice that express a model therapeutic gene h*EPO* under the control of Ig regulatory elements. Applying transgenic mice allowed us to test different vector cassettes and examine the tissue specificity and site dependence of the integrated human transgene. In addition, these mice provide a convenient and reproducible source of transduced cells to test our therapeutic protocol without the need for *in vitro* transfection. We have shown that the Eμ-based cassette directs expression of hEPO to lymphocytes including immature T cells, whereas the λ1-based cassette drives expression in activated B cells in a copy-number-dependent fashion as expected (A.M. and N.D., unpublished data) (32). hEPO was secreted efficiently by mouse B lymphocytes and was biologically potent. Clinically relevant levels of hEPO were achieved after adoptive transfer and antigen stimulation of λ1-hEPO transgenic B lymphocytes that carry only four copies of the transgene per cell. We show that hEPO secretion by donor cells is maintained over several cycles of antigen challenge in normal recipients. This effect was not seen when the recipients were injected with PBS only. Preliminary studies suggest that intravenously injected donor cells homed to the lymphoid organs rapidly, within 2 h after cell transfer (data not shown). When transferring separated PE-specific B cells, we did not generally see examples of hEPO production through nonspecific bystander help (Fig. 2). Transferring polyclonal B cell populations of PE-immunized spleen, however, in a single case, we saw a gradual rise in the recipient's hematocrit level without a requirement for specific antigen PE (data not shown). This incident may have arisen from the activation of transgenic B cells specific for an unrelated antigen, although we cannot rule out a polyclonal expansion of donor cells through bystander stimulation. Further studies will be required to determine whether the on/off properties (and specificity) of this type of B cell expansion is amenable to safe manipulation. However, we caution that additional strategies to moderate or eliminate transferred cell populations (e.g., by introducing conditional suicide genes such as HSV-TK, as described in refs. 33 and 34) will be required.

A second potential problem in applying the proposed strategy is to optimize conditions for the *in vitro* transduction of antigen-specific peripheral B lymphocytes with therapeutic cassettes. At present, this strategy would rely on viral vectors capable of delivering relatively large transgenes (35), because alternative nonviral methods do not provide efficient and stable gene integration in lymphocytes. Recent reports on the adverse effects of using retroviruses for clinical trials in gene therapy (36) emphasize the safety problems associated with the use of these vectors. However, future improvements in corrective strategy and in vector design (37) may limit the risk of dangerous insertion events while allowing efficient transduction (38).

A third important issue of gene therapy strategies aimed at replacing missing serum proteins is the potential of the deficient individual to mount an immune response to the transgene product or the vector. Using the mouse model system described here, we did not detect any anti-hEPO immune responses over a 3-month period (Figs. 2 and 3), and no antibodies against hEPO were detected in the recipients' sera even after repeated antigenic restimulation of the adoptively transferred λ1-hEPO B lymphocytes (data not shown). This result was encouraging, although it may reflect the experimental protocol we used (in particular the lack of a viral vector) and the mouse strain in which these experiments were performed. C57BL/6 mice have been shown to tolerate foreign transgene-encoded proteins such as human α-1 antitrypsin, human factor IX, and hEPO (refs. 39–41; challenged in ref. 42). Although a lack of anti-hEPO immune responses cannot be assumed in other strains or species, it is noteworthy that presentation by B cells often leads to T cell tolerance (43–45), thus raising the possibility of a potential tolerogenic expression of hEPO in λ1-hEPO transgenic B lymphocytes. Further studies will be necessary to evaluate this issue.

In summary, we show compelling evidence that antigen-specific B lymphocytes can be used to deliver clinically significant levels of therapeutic proteins in an antigen-regulated fashion *in vivo* for prolonged periods of time. We demonstrate that this strategy can be applied to correct severe anemia in EPO-deficient mutant mice. In its present form, this approach is suitable for the delivery of biologically active factors in chronic conditions in which regulation is required over long periods of time but where the rapidity and precise level of the response is not critical. It is likely that the range of secreted biomolecules (and diseases) for which this strategy could be applied will increase as knowledge of the human proteome extends.

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