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Antigen-Specific IgG Responses from Naive Human Splenocytes: In Vitro Priming Followed by Antigen Boost in the SCID Mouse[1]

Peter Brams,[2]* Mai-Lan Nguyen,* Soulaima Chamat,* Ivor Royston,† and Phillip R. Morrow‡

High titers of Ag-specific human IgG were consistently achieved in SCID mice reconstituted with human splenocytes that had been primed with Ag in vitro and then boosted with Ag after engraftment into SCID mice. Specific human IgG titers in the hu-SPL-SCID mice reached approximately 1:4 × 10^5 when the mice were immunized with a neo-antigen, whereas titers reached 1:2 × 10^6 when recall responses were induced. Booster immunizations with Ag 21 days after the initial in vivo boost further enhanced this response, and specific human IgG titers of 1:17 × 10^6 were achieved. This represented an essentially monospecific IgG population. These responses were $CD4^+$ T cell dependent. In addition, affinity maturation of the human Ab responses was observed. Spleens of hu-SPL-SCID mice with Ag-specific titers ≤1:1 × 10^6 were often significantly enlarged and often displayed visible tumors. Fourteen of sixteen B cell tumors removed from spleens of five such hu-SPL-SCID mice, produced Abs that were specific for the immunizing Ags. From such tumor, cloned cell lines were established. One such mAb, MLN-7 (γ1,κ), was raised to tetanus toxoid and had no identified cross-reactivity. *The Journal of Immunology,* **1998, 160: 2051–2058.**

Human mAbs have therapeutic potential against infectious diseases (1–3) and cancer (4–10). However, attempts to isolate high affinity human mAbs have met with limited success, mainly due to the unavailability of appropriately primed and activated B cells. Some success has been reported using recall Ags. The first Ag-specific human hybridoma described in the literature was generated from the cells of a SRBC-immunized and splenectomized Hodgkin's donor (11), and human mAbs to tetanus toxoid (TT)[3] (12) and to respiratory syncytial virus fusion protein (2) have been generated from cells isolated through combinatorial library generation from vaccinated/naturally immune donors. Gorny et al. (1) used in vitro EBV transformation of donor cells to isolate seven anti-HIV gp120 mAbs. All of these approaches have succeeded only with recall Ags and are highly work intensive (e.g., Gorny et al. reported having to screen 14,329 clones). It has been suggested that Abs to neo-Ags can be obtained using very large repertoire combinatorial gene libraries. This can be done either directly from the naive repertoire (13) or through epitope imprint selection (14) from mouse mAbs. However, only Abs of relatively low affinity have been isolated from such libraries (13). Three interesting alternatives have been published recently. One approach is to use immunized monkeys as a source of near human Abs and then humanize the Ab (15). The second is to use transgenic mice, which express only human Ig genes (16). The third approach, exploiting the sex difference, is to prime human female spleen cells in vitro with prostate-associated Ags (17). We decided to explore use of the SCID mouse as host for human lymphocytes (18) to induce human cognate immune responses of levels applicable for immortalization.

Activation of human B cells ex homine may be performed in vitro (19–21) or in SCID mice (22) reconstituted with human lymphocytes (18, 23, 24). Generally, in vitro priming can be done only once and is applicable only for inducing primary responses or enhancing recall responses. Such responses have hitherto generated Abs of relatively low affinity that would have marginal therapeutic value (19, 25). The most common human-SCID model with PBLs, the so-called hu-PBL-SCID, has been used to generate TT-specific IgG reciprocal titers up to 5 × 10^5 with cells from TT-vaccinated donors (12, 26). However, this approach cannot be used for induction of primary responses (18). Two other grafted SCID mouse models have also been described in which secondary responses from naive cells were induced (23, 24); McCune et al. (23) created the necessary environment by surgically inserting, under the kidney capsule of a SCID mouse, human fetal liver and thymus tissue as the source of immature B and T cells, respectively. Lubin et al. (24) used human bone marrow cells. These two systems have limited application, however, since the former makes use of scarce and controversial fetal tissue, and both are technically demanding and require approximately 4 mo for the establishment and maturation of the human B cells. Also, the resulting responses were weak compared with antisera from primed donors; the highest reciprocal titers achieved were above 10^3.

Recently, a novel approach for priming and boosting naive human spleen cells in vitro, resulting in significantly enhanced IgG responses, was published (20). This was achieved by using a 3-day priming step, followed by a resting period of 7 days, and then subjecting the cells to a second Ag challenge. During the second Ag challenge, Ag presentation was replenished by adding fresh autologous cells. In this report, we describe how performing the second Ag challenge in engrafted SCID mice results in high titers of Ag-specific human IgG to both neo- and recall Ags. We also show that human cells from such hu-SPL-SCID mice can be used

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[3] Abbreviations used in this paper: TT, tetanus toxoid; HRP, horseradish peroxidase; RF10, RPMI with 10% FCS; hu-SPL-SCID, SCID mouse reconstituted with human spleen cells.

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to generate high affinity human mAbs with potential therapeutic application.

Materials and Methods

Reagents

Horse spleen ferritin (Cat. No. F 4503) was purchased from Sigma, St. Louis, MO. Protein A-Sepharose (Cat. No. 17-0780-01) is from Pharmacia (Uppsala, Sweden). Human IgG and IgM standards (Cat. No. 55908 and 55916, respectively) are Cappell products (Durham, NC). Goat anti-human IgM, goat anti-human IgG, horseradish peroxidase (HRP)-conjugated goat anti-human IgM, HRP-conjugated rabbit anti-human IgG (mouse IgG absorbed), and HRP-conjugated goat anti-mouse IgG (human IgG absorbed) (Cat. No. 2020-01, 2040-01, 2020-05, 2040-05, 6145-05, and 1010-05, respectively) were all from Southern Biotechnology Associates (Birmingham, AL). The isotyping kit was from AMAC, Inc., Westbrook, ME (Cat. No. 0300). Mouse anti-TT C fragment, clone 49.4 (Cat. No. 1348 655) was from Boehringer-Mannheim (Indianapolis, IN). IL-2, IL-4, and IL-6 (Cat. No. 202-IL, 204-IL, 206-IL, respectively) were from R&D Systems (Minneapolis, MN). TT was from Wyeth-Ayerst Laboratories Inc., Marietta, PA. CFA (Cat. No. F-5881) was from Sigma, and Imject Alum (Cat. No. 77160) was from Pierce, Rockford, IL.

Mice

C.B-17 *scid/scid* (SCID) mice were purchased from Harlan Sprague Dawley (Indianapolis, IN). SCID mice are unable to recombine the genes that code for the variable and the constant regions of their B and T cell Ag receptors. Only nonleaky mice with murine IgG levels below 100 ng/ml were used in this study. The mice were kept under sterile conditions in ventilated microisolator cages at the animal facilities of the Medical Biology Institute (La Jolla, CA) or at IDEC Pharmaceuticals (San Diego, CA), both of which meet the National Institutes of Health guidelines as described in the "Guide for Care and Use of Laboratory Animals." All bedding, food, water, containers, and utensils were autoclaved or irradiated before use.

Spleen processing

Spleens from idiopathic thrombocytopenic purpura patients or accident victims were provided by the University of San Diego, California Tissue Bank or the Cooperative Human Tissue Network in Columbus, OH. Donors were screened for HIV and hepatitis and were between 18 and 55 yr of age. The spleens were processed immediately upon arrival, essentially as described (19). Briefly, pieces of spleen were pressed through a stainless steel mesh. Single cells were separated from fragments by letting the fragments settle for 1 min and then harvesting the supernatant. The cells were then collected by centrifugation at $250 \times g$ for 5 min. RBC were lyzed in 0.155 M NH_4Cl for 1 to 2 min. The resulting enriched mononuclear cell suspension was resuspended at a concentration of 4×10^8 cells/ml in ice-cold RPMI 1640 containing 30% FCS and 10% DMSO (Sigma; Cat. No. D-2650), frozen, and stored in liquid nitrogen until use. Spleens were screened for in vitro Ag activity before use (see below). Optimal Ag concentration was determined as described previously (19, 28).

In vitro immunization and cell culture

In vitro immunization was done essentially as described previously (19, 20, 28). Briefly, spleen cells were thawed and washed in RPMI 1640 containing 2 mM L-glutamin, 1 mM sodium pyruvate, nonessential amino acids, and 15 mM HEPES, pH 7.4 (RPMI). The cells were resuspended at 3×10^6 cells/ml in RPMI containing 10% FCS (RF10) or 10% human AB serum (Scantibodies, San Diego, CA, Cat. No. 3SM648). Responses by some spleens to ferritin was enhanced by addition of IL-2. Where such spleens were, used IL-2 was added at 25 IU/ml (28). This cell suspension was plated out at 2 ml/well into the wells of a 24-well cell culture plate. Ag at various concentrations was added to the cultures. Supernatant taken on day 7 was tested in ELISA (see below) for Abs to the immunizing Ag. The Ag concentration resulting in the highest response was subsequently used throughout. Only spleens that responded to the immunizing Ag were used. Cells destined for transfer into SCID mice were incubated the described time with the optimal Ag concentration, resuspended in RF10, and washed once before transfer to SCID mice.

Transfer of human spleen cells to SCID mice and in vivo boosting

Between 2.5×10^6 and 5×10^7 human spleen cells were injected into SCID mice i.p. in 0.1 to 0.2 ml RF10. The hu-SPL-SCID mice were immunized/boosted i.p. with 10 to 15 μg Ag in $\leq$0.15 ml PBS 1 to 7 days later. The Ag was mixed 1:1 with adjuvant (alum for TT and IFA for ferritin). In some experiments, freshly thawed, 2000-rad irradiated, autologous spleen cells were transferred i.p. to established hu-SPL-SCID mice either 1 day before the regular Ag boost or on the same day of a reboost. The exact designs and the numbers of cells transferred with these protocols are indicated in the text. Blood (0.2 ml) was collected by retro-orbital sinus rupture. Serum was collected from clotted blood and frozen until use.

Rescue of B cells producing Ag-specific Abs

hu-SPL-SCID mice with titers $\leq 1:1 \times 10^6$ often developed EBV-transformed B cell tumors on their spleens. Spleens from such hu-SPL-SCID mice were aseptically removed following cervical dislocation. Tumors were recovered, and single-cell suspensions were prepared by sieving the cells through a mesh. The cells were then dispensed into 96-well plates and scored for TT-specific IgG production. Cells from positive wells were cloned by limiting dilution.

ELISA

ELISAs were performed essentially as described previously (19). Briefly, ELISA plates (Immulon 1, Dynatech Laboratories, Chantilly, VA, Cat. No. 0110103455) were coated with ferritin (50 μl/well at 10 μg/ml), TT (50 μl/well at 2 μg/ml), goat anti-human IgM, or IgG (50 μl/well at 2 μg/ml) in 0.05 M sodium carbonate buffer, pH 9.3, for 2 h at 37°C. The plates were washed and blocked with RF-10 before adding 50 μl of serum diluted with RF10 and incubating for 2 h at 37°C. Sera from mock-boosted hu-SPL-SCID mice were used as negative controls. Binding of human IgM and IgG was revealed with HRP-conjugated goat anti-human μ- and γ-chain, respectively. The reaction was visualized by the addition of *O*-phenylenediamine (Cat. No. O-1526, Sigma) substrate in a sodium citrate buffer, pH 5.0, containing 0.0175% H_2O_2. The enzyme reaction was stopped by addition of 3 M H_2SO_4 and read at 490 nm.

Quantitation of anti-ferritin activity, performed by comparing serum responses to a purified monoclonal human anti-ferritin IgG Ab, 21-1B-9 (19), was expressed as μg/ml equivalents of this Ab. Anti-TT serum activity was expressed as either the reciprocal of end dilution titer (the dilution at which OD 490 was twice background) or in MLN-7 equivalents (see Fig. 4). Equivalents were defined using the concentration of mAb at which half-maximal binding was observed. Based on the assumption that the Abs in the serum had average affinities similar to those of the mAbs, the total concentration of Ag-specific Abs was estimated by multiplying the appropriate equivalence concentration with the reciprocal serum titer, resulting in half-maximal binding. Half-maximal binding with 21-1B-9 was achieved at 29 ng/ml; half-maximal binding with MLN-7 was achieved at 8.7 ng/ml. Concentration of IgM and IgG in serum was determined by comparing to polyclonal human IgM and IgG standards.

Relative affinity measurements were performed essentially as described by MacDonald et al. (27). Briefly, Ag was bound to plastic plates, followed by washing, blocking, and incubation with primary Ab, as described above. KSCN (Sigma, Cat. No. P-2713, 100 μl) dissolved in PBS in concentrations ranging from 1 to 5 M was added and incubated for 15 min. The cyanate salt was flicked out and the plate washed 5 times. Secondary goat anti-human IgG HRP-conjugated Ab was then added, and the presence of primary Ab was developed with o-phenylenediamine as described above.

Statistics

Statistical analysis was done using the Excel Data Analysis program (Microsoft Excel version 4, Microsoft, Seattle, WA). Mean values are arithmetic means for single experiment calculations and geometric means for multiple experiment calculations. Geometric means were calculated based on transformed data of log base 3 ($\log_3$). The p values reported were based on 95% confidence level and two-tail distribution.

Results

In vitro stimulation of human spleen cells combined with boosting in hu-SPL-SCID

Various protocols for generating human Ab responses with predetermined specificity in SCID mice reconstituted with human splenocytes were tested. One protocol consisted of a direct transfer of nonstimulated spleen cells to SCID mice. A second introduced an in vitro cultivation and priming period of the human splenocytes before transfer into SCID mice. Further reconstitution of hu-SPL-SCID mice with autologous human spleen cells 1 day before boosting was also tested. To determine the requirements for

Table I. *Effect of in vitro cultivation and antigen challenge in vitro/in vivo*[a]

	Protocol					
	Culture		SCID		Response: SCID Sera	
A	Ferritin	Cultivation time	Ferritin	Fresh cell transfer	Human IgG (μg/ml)	Ferritin binding (μg/ml)*
1	NA	0	+	−	1.8 ± 1	<0.1
2	−	3	−	−	33 ± 10	<0.1
3	−	10	+	−	15 ± 5	0.3 ± 0.1
4	+	3	−	−	62 ± 19	<0.1
5	+	3	+	−	70 ± 31	3.8 ± 1.3
6	+	10	+	−	10 ± 1	2.9 ± 0.4
7	+	3	+	+	84 ± 29	11.6 ± 2.3
8	+	10	+	+	21 ± 3	5.1 ± 0.6

B	Culture TT	SCID TT	Human IgG titer/$\log_3$	TT binding titer/$\log_3$†
1	NA	+	7.988 ± 1.26	<4.192
2	−	−	11.963 ± 0.832	<4.192
3	−	+	11.904 ± 1.112	10.376 ± 1.065
4	+	−	11.234 ± 0.430	8.120 ± 1.759
5	+	+	12.076 ± 0.766	12.672 ± 0.737

[a] Responses by human splenocytes subjected to Ag challenge either in vitro, in vivo, or both were measured after transfer to SCID mice. Responses to both a neo-antigen (A), horse ferritin, and a recall Ag (B), TT, were measured. A. Five SCID mice in each experiment were engrafted each with 5×10^6 human spleen cells from spleen NN, that had been subjected to in vitro cultivation for 0, 3, or 10 days with (%) or without (−) 1 μg/ml horse ferritin. The hu-SPL-SCID mice were boosted at day 11 with 10 μg of ferritin (+) in IFA or IFA alone (−). In experiments 7 and 8, 2.5×10^6 in vitro primed cells were transferred at the indicated day, followed by a similar number of fresh, autologous cells at day 10. *, Ferritin-specific responses were measured parallel to a human anti-ferritin Ab, 21-1B-9, and are recorded as micrograms per milliliter of this antibody, resulting in similar responses (OD_{490} readings). Data are given as means ± SD. Sera were taken 29 days after the start of the experiment. B. A compilation of five experiments, with four different spleens. A minimum of four mice was used in any group in all experiments. Each SCID mouse was engrafted with $\sim 2 \times 10^7$ cells that had been cultivated in vitro with (+) or without (−) optimal amounts of TT (see *Materials and Methods*). Seven days later, the resulting hu-SPL-SCID mice were then immunized with TT emulsified in alum (+) or with PBS emulsified in alum (−). TT-specific responses are reported in $\log_3$ titers (3 was the dilution factor) as geometric means ± SD. *p* values were calculated based on the geometric means ± SD. †, Dilutions were started at 1:100; therefore, nonresponders have titers below $100 = 4.192$ $\log_3$. NA, not applicable.

Table II. *Effect of in vitro IL-2 on ferritin-specific IgG responses from hu-SPL-SCID mice*

	Human IgG Responses	
Experimental Conditions[a]	Total (μg/ml)	Ag-specific (μg/ml)
−IL-2	75 ± 56	2.3 ± 2.0
+IL-2	110 ± 70	7.8 ± 2.1

[a] Cells (5×10^7) from spleen K, primed in vitro with ferritin with or without 25 U/ml IL-2 were transferred to each SCID mouse. Each SCID mouse (five mice in each experiment) was immunized with 10 μg of ferritin in IFA. The concentration of total IgG and ferritin-specific IgG, expressed as 21-1B-9 equivalents, was determined in ELISA as described in *Materials and Methods*. Data are given as means ± SD. Sera were taken 29 days after start of experiment.

generating Ag-specific IgG responses to a neo-Ag, we used an established Ag system, horse ferritin (19, 20, 28). The results (see Table I*A*) show that in vitro cultivation was essential to obtain robust reconstitution of the mice, gauged as amount of human IgG in the hu-SPL-SCID serum. Ag challenge both in vitro and in vivo was necessary to obtain the highest levels of Ag-specific Ab responses. Furthermore, addition of fresh autologous spleen cells just before Ag boosting significantly increased the Ag-specific Ab responses.

The requirement for in vitro spleen cell cultivation was confirmed in a different Ag system using the recall Ag TT (Table I*B*). In this system, Ab titers below 1:100 were obtained when hu-SPL-SCID mice, reconstituted with noncultivated human spleen cells, were immunized with TT. These results correlate with those reported in the literature in comparable hu-PBL-SCID chimera studies (18, 23, 24). By adding an Ag challenge of the cells in vitro, Ag-specific IgG titers increased by a factor of more than 11,000, whereas the total IgG concentrations increased only by a factor of 90. Again, the highest Ag-specific responses were obtained when the human cells were challenged with Ag both in vitro and in the hu-SPL-SCID mice. There was no statistically significant difference at the 95% confidence level between groups 2 through 5 in terms of IgG production, but *p* values for comparisons of the same groups in terms of anti-TT responses were all below 0.01.

The isotype of the Ag-specific Ig was overwhelmingly IgG; Ag-specific IgM titers were >100-fold weaker than IgG titers, whether neo-Ag or recall Ag was used (not shown). This finding is in contrast to what is observed in vitro (17, 19, 20, 28), where responses are overwhelmingly IgM.

Responses to ferritin from some spleens were enhanced by adding IL-2 in vitro. In a representative experiment in which we used cells primed in vitro ± 25 U/ml IL-2, we found that subsequent hu-SPL-SCID serum IgG levels were not different whether the human cells had been cultivated with or without IL-2, whereas anti-ferritin levels differed (see Table II), with a p value < 0.01. IL-2 in vitro did not significantly enhance induction of recall responses relative to the total IgG levels (not shown).

Spleens of the highest responders were significantly larger than the spleens of the weaker responders. In an experiment in which eight SCID mice were repopulated with in vitro-cultivated cells, five of the resulting hu-SPL-SCID mice were boosted with TT and 3 were given a mock boost. The total IgG and anti-TT IgG reciprocal titers of the sera throughout the experiment are shown in Figure 1*A*. The spleens from all eight mice were removed on day 35 after boost and placed in the wells of a 24-well tissue culture plate (see Fig. 1*B*). The three hu-SPL-SCID mice with the large spleens (average, 345 ± 46 mg) had reciprocal anti-TT IgG titers $>1 \times 10^5$, vs reciprocal titers of $<1 \times 10^4$ for the two remaining TT-boosted mice with normal size spleens. The nonboosted mice had reciprocal anti-TT titers $\leq 10^2$ and normal size spleens. The average size of the five normal size spleens was 37 ± 10 mg. The anti-TT titers were significantly different in the mice with large spleens compared with titers in the mice with normal size spleen (p value < 0.01 for all data points), whereas the total IgG titers were not. Tumor masses were often found on the large spleens (see below).

The results described above have all been confirmed with at least two different Ags and at least three different donors.

Induction of Ag-specific IgG responses are T cell dependent

To determine whether the Ag-specific responses were T cell dependent, four hu-SPL-SCID mice were given a dose of a murine anti-human CD4 Ab 1 day before immunization with TT. The anti-CD4 Ab, 5A8, has $CD4^+$ T cell-neutralizing activity. A parallel group not treated with 5A8 functioned as a control. Serum concentrations of total human IgG and anti-TT IgG were determined 25 days after immunization (Fig. 2). The results show that the TT-specific titers in the group treated with anti-CD4 was ~350-fold lower than the TT titers in the control group (p value of 0.001 based on the $\log_3$-transformed data), whereas the levels of total IgG was reduced to approximately one-third (p value of 0.29). This experiment has been repeated twice.

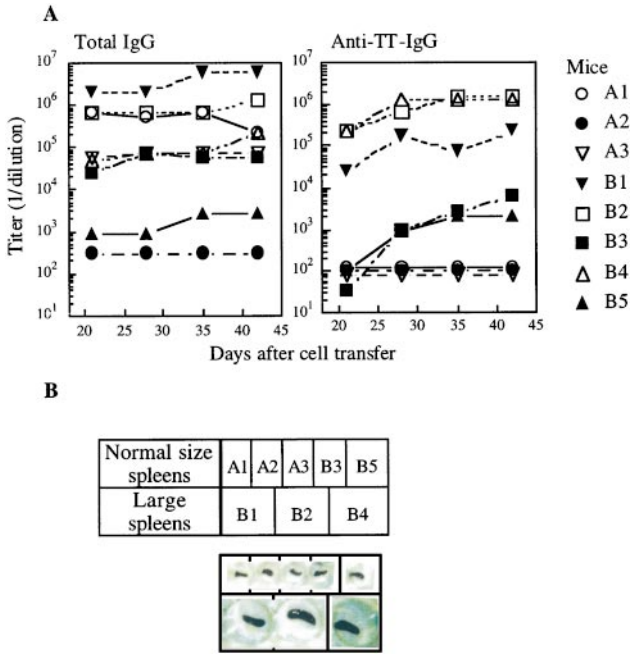


FIGURE 1. Eight SCID mice each received 1.2×10^7 human spleen cells, spleen 29, that had been cultivated in vitro in human AB serum. The three mice in group A received cells that were cultivated without TT, whereas the mice in group B received cells that were cultivated in the presence of 1 μg/ml of TT. The mice in group B were each boosted with 10 μg of TT resuspended in alum 7 days after cell transfer. Serum was taken weekly from all of the mice and tested for anti-TT IgG titers and total IgG titers. Thirty-five days after TT boost, the mice were killed and the spleens were taken out for visual inspection. *A*, Total IgG titers and anti-TT titers are depicted for each hu-SPL-SCID mouse. *B*, Spleens taken out on day 35 after TT boost from all eight mice are shown under a grid identifying each spleen. The five normal size spleens are arranged in the wells of a 24-well plate, while the three large spleens are arranged in the wells of a 6-well plate.

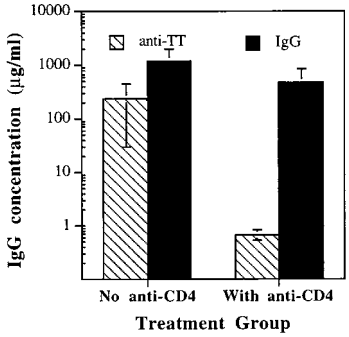


FIGURE 2. Eight SCID mice each received 2.5×10^7 cells from spleen 42, which had been cultivated in vitro for 3 days in the presence of 1 μg/ml TT. Four of these hu-SPL-SCID mice each received four injections of 200 μg 5A8, anti-human CD4, with a 3- to 4-day interval, starting 6 days after cell transfer. All mice were boosted with 10 μg TT/mouse resuspended in alum, 7 days after cell transfer. Anti-TT MLN-7 (Fig. 4) equivalents and total IgG levels were measured in serum taken 25 days after boosting and are displayed as the means ± SD of the concentration of the four sera of each group.

Affinity maturation of human anti-ferritin Abs in hu-SPL-SCID serum

To determine whether affinity maturation of the human anti-ferritin Abs was occurring in the hu-SPL-SCID mice during a response, we applied the KSCN elution resistance method (see *Materials amd Methods*) to serum taken at different time points after Ag boost. The result of one such experiment is shown in Figure 3. The profiles show that ~80% of the measured responses in the sera taken 13 and 28 days after cell transfer are of low relative affinity. The proportion of high affinity, ferritin-reactive IgG present in the serum after 52 days increased to 70%. The Abs that remained bound to ferritin at levels >0.6 M KSCN could not be eluted off with 5 M KSCN. Comparable results were obtained for three other mice examined in the same experiment. These results indicate that B cells producing Abs of relatively high affinity to the immunizing Ag expand over time, potentially enabling preferential immortalization of high affinity mAb production.

Repeated Ag boost of hu-SPL-SCID mice

To test whether repeated in vivo Ag/adjuvant boosts would affect ongoing responses to Ag, hu-SPL-SCID mice responding to Ag after the first boost were given a second Ag/adjuvant challenge 21 days later. This was tested two ways: 1) by simply boosting hu-SPL-SCID mice again, essentially as described by Lubin et al. (24); and 2) by boosting hu-SPL-SCID mice concomitantly given fresh, irradiated, autologous human spleen cells (Fig. 4). The results show that simple reboosting resulted in increased total IgG levels rather than in enhanced specific IgG titers (Fig. 4*A*). Restimulation in the presence of fresh autologous cells, however, resulted in enhanced Ag-specific IgG titers (Fig. 4*B*). Total IgG levels, as well as anti-TT IgG, were different between the two groups from day 27, the first bleed after restimulation, through day 41 (see table in Fig. 4*C*). One of the mice subjected to simple restimulation showed an increase of total IgG of almost 55-fold, whereas Ag-specific IgG titers decreased to approximately one-fifth of that before restimulation (Fig. 4*D*). One of the mice given fresh cells concomitantly with the boost showed a marginal increase of total IgG titer, whereas the Ag-specific IgG titers increased ~10-fold by day 30 (Fig. 4*E*). Half-maximal responses to TT on day 34 in the two mice shown were at 4.5 μg IgG/ml and 10 ng IgG/ml, respectively. A similar trend was seen in hu-SPL-SCID mice reconstituted with cells from a different donor and immunized with ferritin (data not shown).

The ratio of Ag-specific human IgG to total human IgG in the serum of the hu-SPL-SCID mouse depicted in Figure 4*E* was 1 of every 1.15 Abs (Fig. 5*A*), expressed as MLN-7 equivalents (see below). This level was reached with two of the five mice. The highest specific titers measured in hu-SPL-SCID mice immunized

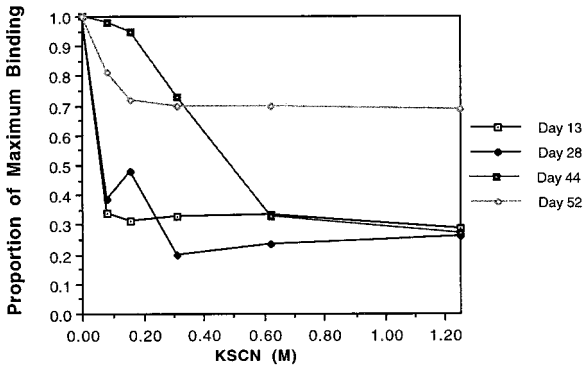
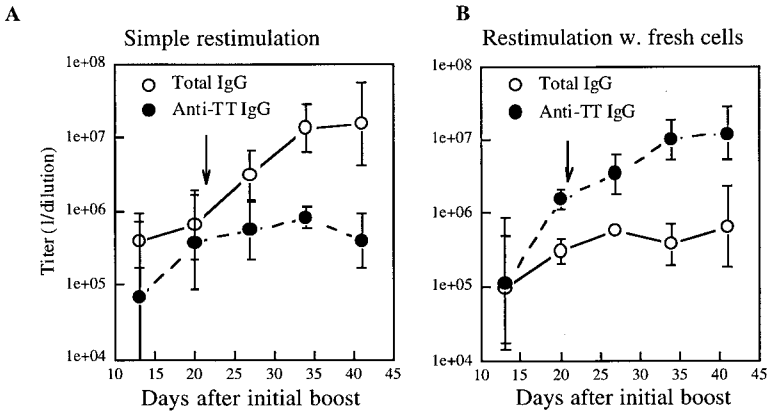


FIGURE 3. Relative affinity changes with time in hu-SPL-SCID mice. Mice received 45×10^6 cells from spleen Z, which had been primed with ferritin for 3 days in vitro in the presence of 25 U/ml IL-2. The hu-SPL-SCID mice were boosted with ferritin in IFA on day 7 after cell transfer.



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Parameter	P values: Group 1 vs. Group 2 Days after initial boost Day 13	Day 20	Day 27	Day 34	Day 41
Total IgG	> 0.05	> 0.05	< 0.01	< 0.01	< 0.01
Anti-TT IgG	> 0.05	> 0.05	= 0.01	< 0.01	< 0.01

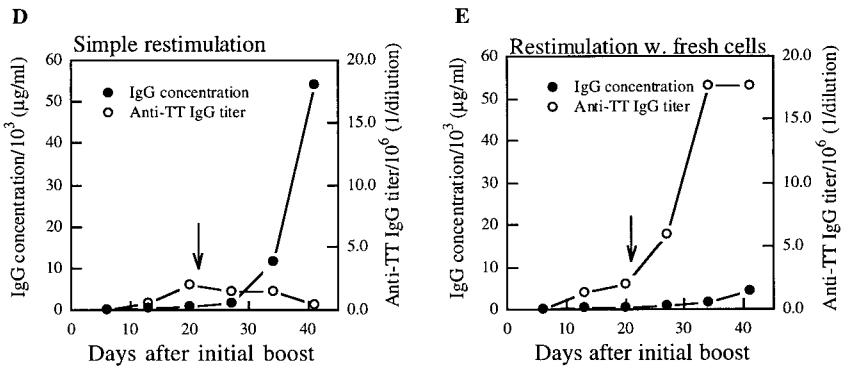

FIGURE 4. Cells from spleen 29 were primed with 10 μg/ml TT in vitro for 3 days before transfer. Ten mice, five in each group, were immunized 7 days after transfer of 1.2×10^7 cells/mouse with 10 μg TT in PBS. On day 21 after immunization, all of the mice received a booster of 10 μg each of TT in alum. The five mice in group 1 did not receive anything else, whereas the five mice in group 2 also received 0.5×10^7 spleen 29 cells each. These cells were irradiated with 2000 rad just before injection. Results from the two groups are compared. *A*, Average levels of IgG and anti-TT titers are shown throughout the experiment for group 1. *B*, Average levels of IgG and anti-TT titers are shown throughout the experiment for group 2. *C*, Significance levels, *p* values, comparing total IgG levels and anti-TT IgG levels between group 1 and group 2 based on $\log_3$ values (dilution factor) of data shown in *A* and *B*. One representative animal is shown for each experiment in *D* and *E*. *D*, In this experiment, the mouse received TT in alum only on day 21 after immunization. *E*, In this experiment, the mouse received TT in alum plus irradiated autologous spleen cells day 21 after immunization. The levels of human IgG were determined in ELISA either as end-dilution titers or relative to a human IgG standard. The levels of anti-TT IgG responses were measured as end-dilution titers. The scales in *A* and *B* are Log scales, while those in *C* and *D* are proportional. Arrows indicate time of restimulation.

with ferritin as Ag was 1 of every 12 Abs (Fig. 5*B*) using 21-1B-9 as standard.

Generation of Ag-specific human mAbs

From the spleen of the hu-SPL-SCID mouse with a reciprocal anti-TT titer of 17×10^6 depicted in Figure 4*E*, we isolated an anti-TT human mAb-producing cell line, MLN-7. Half-maximal binding in ELISA by this Ab was 8.7 ng/ml as compared with the mouse anti-TT Ab, 49.4, which had half-maximal binding at 25 ng/ml (Fig. 5*A*). The isotype of MLN-7 was determined to be $\gamma 1,\kappa$. No binding by the Ab to a series of human cell lines, as measured by flow cytometry, or to a series of selected Ags, measured by ELISA, was identified (data not shown).

A summary of the yield of immortalization attempts in four independent experiments is shown in Table III. Fourteen of sixteen tumors produced Ab to the immunizing Ag, and of these, three had a half-maximal response at a lower concentration than commercially available murine mAbs. MLN-7 came from spleen 29. The two Abs to respiratory syncytial virus fusion protein are characterized in detail elsewhere.[4] The tumor masses contained between 3 and 30×10^7 cells of which up to 35% were CD20 positive by flow cytometry (not shown).

[4] Chamat, S., E. E. Walsh, D. Anderson, L.-Z. Pan, S. Dillon, S. Demuth, J. Ochi, S. Shuey, and P. Brams. Two human monoclonal antibodies isolated from spontaneous tumors of Hu-SPL-SCID mice and specific for respiratory syncytial virus fusion protein, display broad in vitro neutralizing activity. *Submitted for publication.*

Discussion

One of the major limitations of generating human mAbs of therapeutic value has been obtaining a source of appropriately primed human B cells for immortalization. Ag-specific responses from human splenocytes induced solely by in vitro means are relatively weak (20, 28). As can be seen from our data and from data published elsewhere (18, 23, 24, 26, 27, 29–34), Ab responses from hu-SPL-SCID mice established from cells transferred without an in vitro cultivation step and responses from hu-PBL-SCID mice both are relatively weak as well. In this study, we demonstrate that secondary (i.e., IgG) human Ab responses can be generated to a neo-Ag ex homine from human spleen cells by combining in vitro priming followed by transfer of these cells into a SCID mouse host, which then is boosted with Ag emulsified in a strong adjuvant. This protocol also applies to recall Ags. One interpretation is that the in vitro immunization drives the human B cells to a stage of differentiation at which they are better able to not only survive in the SCID mouse host but also to proliferate and undergo somatic hypermutation more efficiently. Furthermore, it does appear that the in vitro environment is better at supporting primary responses as a prerequisite for generating secondary responses to neo-Ags subsequently in the hu-SPL-SCID mouse. For responses to ferritin, a neo-Ag, up to 1 of every 12 hu-SPL-SCID serum Abs could be directed to the immunizing Ag, while for recall responses virtually all Abs could be directed to the Ag. Also, these responses could be

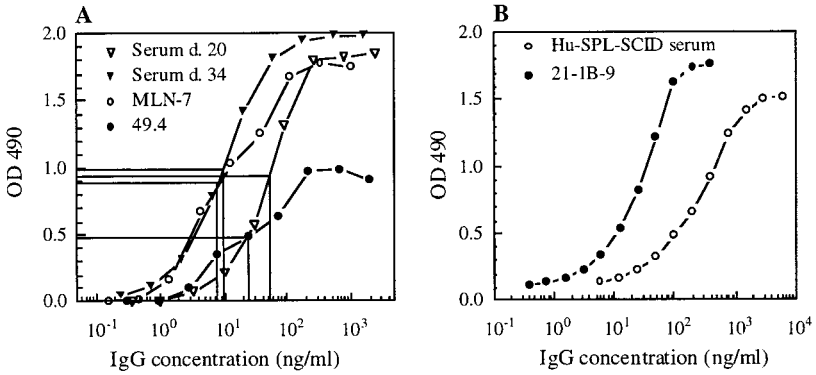

FIGURE 5. Ag-specific human IgG titers in hu-SPL-SCIDs. *A*, The anti-TT IgG activity of the sera described in Figure 4, group 2, day 21 and day 34, were evaluated in a dilution series along with a human anti-TT mAb, MLN-7, that was isolated from a tumor recovered from this hu-SCID-mouse. Reactivity to TT by a murine anti-TT mAb (49.4, Boehringer-Mannheim) was also tested. Half-maximal binding to TT was achieved at 50 ng/ml and at 9.8 ng/ml for the two sera, respectively, and at 8.7 ng/ml and at 25 ng/ml for MNL-7 and 49.4, respectively. *B*, Serum anti-ferritin response from a hu-SPL-SCID mouse boosted with 10 μg ferritin in alum. Serum was taken 20 days later, and the human IgG concentration was determined to be 1.25 mg/ml. The anti-ferritin IgG activity was tested in a dilution series along with a human anti-ferritin mAb, 21-1B-9 (18). Half-maximal titer of 21-1B-9 was 29 ng/ml, whereas half-maximal responses of the hu-SPL-SCID serum was 350 ng/ml.

immortalized. The Ag directed responses were $CD4^+$ T cell dependent, as has been shown elsewhere for similar models (29, 30). However, with our protocol, in which the reconstitution event and the treatment-immunization events have been separated by 7 days, only Ag-driven responses, and not the background, were affected by the anti-CD4 Ab, supporting our notion that this protocol is uniquely applicable for in vivo studies of regulation of cognate immune responses. These data also show that the use of human spleen cells and in vitro Ag priming significantly enhances Ag-specific responses and reconstitution compared with the hu-PBL-SCID model.

The presence of IL-2 during the in vitro priming period was found to be a significant factor when the target was a neo-Ag. The fact that we did not find exogenous IL-2 to be a factor for recall responses is likely to be a product of our spleen selection procedure. We have previously determined that IL-2 is essential for the generation of all Ag-specific responses in vitro, as IL-2-neutralizing Abs abrogate Ag-directed responses (28). However, we have yet to transfer cells cultivated with IL-2-neutralizing Abs to SCID.

One of the advantages of this model is the reproducibility of responses from different donors. The titers obtained in the experiments shown in Table I*B* and in Figure 2, control group (no anti-CD4), and the titers observed in the experiment shown in Figure 4, *D* and *E*, before restimulation, are all highly comparable: ~1 mg/ml IgG and between 1 and 3×10^6 in reciprocal titer (the 1200 μg/ml mAb equivalents shown in Figure 2, control group, equate to a reciprocal titer of $\sim 2.5 \times 10^6$). Also, the conclusions reached in Table I, *A* and *B*, are entirely parallel even though different donors and two different Ags were used.

The highest Ag responders were found to have enlarged spleens. In three different experiments with >60 hu-SPL-SCID mice, we never saw a large (>5× the average normal weight) spleen in mice with reciprocal Ag-specific titers under 10^5. All of the mAbs isolated from these mice were produced by tumors cut from large spleens. We have no explanation for the increased size, as some of these large spleens, apart from the tumors, contained <5% human cells and never >35% (not shown).

Results obtained by other groups (31, 32) strongly suggest that human T cells become anergic in the hu-PBL-SCID mouse by day 35. These results imply that reboosting of established responses in the hu-SPL-SCID mouse after a certain time would have little effect. Our data essentially confirm, but also extend, these observations, as reboosting had contrasting effects on the Ag-specific responses depending on the particular protocol used. In hu-SPL-SCID mice that were simply reboosted with Ag in adjuvant 29 days after cell transfer (21 days after initial Ag boost), we observed only a nonspecific effect of the adjuvant, that of noncognate B cell stimulation, i.e., increases in total IgG levels. It is likely that the increase in IgG levels in this group was due to B cell lymphomas activated by the adjuvant, as lymphomas were identified on the spleen of the mice, depicted in Figure 4*D*, although these tumors were not further analyzed. However, by combining an injection of Ag with concomitant transfer of fresh, irradiated autologous cells 21 days after the first in vivo Ag boost (i.e., day 31 after cell transfer), we observed an increase in Ag-specific responses by a factor of 10 to 20 at the same time that levels of total IgG increased only slightly. This would argue that addition of fresh autologous cells at the time of the secondary in vivo Ag boost facilitates cognate B-T cell interactions, since functional T cells are essential for induction of affinity maturation and for induction of Ag-specific Ab responses. The transferred cells were irradiated but, as suggested by Tary-Lehmann et al. (33, 34), we observed that this did not impact Ag presentation or T cell help. Furthermore, it was evident that, despite identified lymphomas from the mouse depicted in Figure 4*E*, from which MLN-7 was established, the serum from this mouse did not show unrestricted IgG production similar to the mouse from the first group. This discrepancy, we

Table III. *Summary of immortalization attempts*[a]

No. of Mice	Ag	No. of Tumors	No. Ag-Specific Tumors	Interesting MAbs	Spleen Designation
2	Fusion protein	4	3	1	Z
1	Fusion protein	5	5	1	Z
1	TI	2	1	0	32
1	TI	5	5	1	29

[a] Summary of immortalizations of B cells with predetermined specificity from hu-SPL-SCID mice stimulated according to the protocol described in this article. Hu-SPL-SCID mice with reciprocal titers $>1 \times 10^6$/ml to the immunizing Ag were sacrificed, and the spleens were investigated for the presence of tumor masses. Tumors were cut out, made into single-cell suspensions, and then cultivated. Supernatants from these cultures were tested for the presence of human Ag-specific IgG by ELISA. Cultures producing Abs that were deemed interesting, based on Ab-binding characteristics, were cloned by limiting dilution. F-protein is the fusion protein from respiratory syncytial virus.

suggest, could be a consequence of the transfer of functional T cells to the mice in the second group, as these T cells might, at least for a period, inhibit nonspecific B cell growth, including that of EBV-transformed cells.

In apparent contrast to the observation discussed above, in which the T cells residing in hu-PBL-SCID mice after day 30 become anergic, relative affinity analysis of the ongoing responses indicated that B cells producing Abs of high affinity were preferentially maintained over those producing Abs of lower affinity, as seen in day 44 and day 52 sera compared with earlier sera. It is likely that this reflects events that have taken place much earlier. B cell responses, as measured here, are the sum of activation, maturation, and accumulated production over a period of time. Once activated through cognate interaction, B cells need only soluble factors or mitogenic stimuli to proceed. This effect likely was also reflected in the potency of the immortalized mAbs described here, where MLN-7 had higher activity than a mouse mAb. The cell that produced MLN-7 was taken from a hu-SCID mouse 41 days after transfer of the nonirradiated lymphocytes, a mouse in which 87% of the Abs in the serum were estimated to be directed against the immunizing Ag. In fact, 5 of 5 Ab-secreting cell lines isolated from this mouse produced anti-TT Ab. It, appeared, therefore, that most if not all of the B cells available for immortalization had been Ag activated. This result also shows that human cells can be retrieved from the spleens of hu-SPL-SCID mice as long as the stimulation of these cells is appropriate.

Isolation of Ag-specific human monoclonal IgG Abs by the approach described here is highly practical for a number of reasons. The immunization protocol routinely results in Ag-specific IgG titers better than $1/10^6$. Immortalization attempts can be limited to the hu-SPL-SCID mice with optimally stimulated B cells and/or with the desired specificity through screening of the sera. Excision of tumors is simple and quick, and from these tumors, 100-μg quantities of Ab can be purified and subjected to limited testing within 2 to 3 wk. Furthermore, since selection of the 3 "interesting" clones, as shown in Table III, was done before cloning of the cell population by limiting dilution, it is not unlikely that some of the other tumors, once nonspecific Ab-producing cells had been excluded, could have sourced other interesting mAbs.

Recently, Greiner et al. (35) showed that the problem of retrieving human lymphocytes from hu-SPL-SCID mice could be overcome by using the NOD/LtSz-*scid/scid* mouse as host, as opposed to the C.B-17-*scid/scid* mouse. This was observed using direct transfer of the human cells without an in vitro cultivation step. Our own data with transfer of in vitro-primed cells to NOD/LtSz-*scid/scid* mice compared with C.B-17-*scid/scid* mice or to SCID/*beige* mice (36) showed no significant difference in serum levels of total or Ag-specific human IgG (data not shown). However, we plan to test this mouse as host for hybridoma generation.

Our results confirm previous findings that human lymphocytes injected into SCID mice can establish themselves and that recall responses can be induced. However, the protocol described here, using spleen cells and combining in vitro priming with an in vivo boost, promotes induction of primary responses as well as recall responses, both of greater amplitude than reported previously. The magnitude of the titers, coupled with the use of splenocytes instead of PBL, may explain why human B cell responses can be rescued and immortalized from these hu-SPL-SCID mice, a step that has eluded previous attempts using hu-PBL-SCID mice (37). The resulting mAbs have affinity and specificity profiles that make them potentially clinically useful. Two human mAbs generated to a viral Ag with the protocol described above have shown in vitro virus-neutralizing activity at equal or lower concentrations than any published murine Ab.[4]

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