Generation of Primary Antigen-Specific Human Cytotoxic T Lymphocytes in Human/Mouse Radiation Chimera

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Severe combined immunodeficient (SCID) mice are increasingly used as hosts for the adoptive transfer of human lymphocytes. Human antibody responses can be obtained in these xenogeneic chimeras, but information about the functionality of the human T cells in SCID mice is limited and controversial. Studies using human peripheral blood lymphocytes (PBL) injected intraperitoneally (IP) into SCID mice (hu-PBL-SCID mice) have shown that human T cells from these chimeras are anergic and have a defective signaling via the T-cell receptor. In addition, their antigenic repertoire is limited to xenoreactive clones. In the present study, we tested the functionality of human T cells in a recently described chimeric model. In this system, BALB/c mice are conditioned by irradiation and then transplanted with SCID bone marrow, followed by IP injection of human PBL. Our experiments demonstrated that human T cells, recovered from these hu-PBL-BALB mice within 1 month posttransplant, proliferated and expressed activation markers upon stimulation with anti-CD3 monoclonal antibody. A vigorous antiallogeneic human cytotoxic T-lymphocyte (CTL) response could be generated in these mice by immunizing them with irradiated allogeic cells. Moreover, anti-human immunodeficiency virus type 1 (HIV-1) Nef-specific human CTLs could be generated in vivo from naive lymphocytes by immunization of mouse-human chimeras with a recombinant vaccinia-nef virus. This model may be used to evaluate potential immunomodulatory drugs or cytokines, and could provide a relevant model for testing HIV vaccines, for production of antiviral T-cell clones for adoptive therapy, and for studying human T-cell responses in vivo.

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MATERIALS AND METHODS

Mice. BALB/c mice were obtained from Olac Farms (Bicester, UK) and SCID mice from the Weizmann Institute Animal Breeding Center (Rehovot, Israel). All mice were fed sterile food and acid water containing wide-spectrum antibiotics.

Conditioning regimen. BALB/c mice were exposed to total-body irradiation (TBI), from a gamma beam 150-A 60Co source at a dose rate of 0.7 Gy/min, with 4 Gy followed 3 days later by 10 Gy (split dose).

Viruses. Wild-type vaccinia virus and the recombinant vaccinia-nef virus were a generous gift of Dr Scott Koenig (MedImmune, Gaithersburg, MD). The viruses were propagated in HeLa cells. The virus was added to HeLa cells, in RPMI medium, at a multiplicity of infection (MOI) of 5 pfu/cell. After 1 hour at 37°C, the virus inoculum was replaced with complete medium. The cells were harvested when they showed maximum cytopathic effect and were then

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lysed by four freeze thawing cycles, to prepare a stock with a titer of between 10^7 and 10^9 pfu/mL.

Preparation and transplantation of SCID bone marrow cells. Bone marrow cells were obtained from SCID mice (4 to 10 weeks old), and recipient mice were injected with 2 to 3 x 10^9 SCID bone marrow cells intravenously (IV) in 0.2 mL phosphate-buffered saline (PBS) 1 day after the last irradiation.

Preparation and transplantation of human PBLs. Buffy coats from normal volunteers were layered onto Lymphoprep solution (Nycomed, Oslo, Norway) and spun at 2,000 rpm for 20 minutes. The interlayer was collected, washed twice, counted, and resuspended in PBS, pH 7.4, to the desired cell concentration. Between 80 and 100 x 10^6 human PBL were injected IP into recipient mice, in 0.5 mL PBS. Control mice did not receive human PBL.

Cells and plasma collection from mouse-human chimeras. Animals were bled from the retro-orbital vein using heparin-coated glass capillaries. PBL were isolated using Lymphoprep, washed with PBS containing 1% bovine serum albumin (BSA), and stained for fluorescence-activated cell sorter (FACS) analysis. Plasma was kept for human immunoglobulin determination. Peritoneal cells were obtained by lavage with 2 to 4 mL PBS. Organs were removed after the animals were killed by cervical dislocation. The organs were cut into pieces and pressed through stainless steel sieves to make a cell suspension in PBS, and cells were then isolated using Lymphoprep.

Phenotypic analysis of human cells. Mouse-human chimeras were constructed as described. Leukocytes were isolated from the peritoneum, blood, spleen, and other internal organs of these chimeras, at various intervals posttransplantation, by separation on a Ficoll gradient. The cells were then analyzed by three-color flow cytometry. The monoclonal antibodies used recognize the following surface molecules: CD45 (common leukocyte antigen), CD3 (pan-T cells), CD4, CD8, CD45RA (naive T cells), and CD45RO (memory T cells). The activation of T cells was assessed by the expression of HLA-DR and CD25 (interleukin-2 [IL-2] receptor). FACS analysis was also performed before and after a 3-day stimulation with OKT3 and/or IL-2, as indicated. A gate was set on all the human lymphocytes (CD45^+), and the expression of IL-2 receptor was monitored separately on CD4 and CD8 T cells.

Proliferation assay. Cells recovered from the peritoneum or spleen of the chimeric mice that received human PBLs from the same donor were pooled and stimulated with OKT3 monoclonal antibody. Cells were then analyzed by three-color flow cytometry. The cells were then harvested with an automatic cell harvester onto glass-fiber filter paper and the radioactivity counted in a liquid scintillation counter. The results are expressed as arithmetic means of CPM/culture ± SE.

Cytotoxicity test. The cytotoxic activity of human T cells was measured using the ^51Cr release assay. Where appropriate, the target cells, consisting of phytohemagglutinin (PHA) blasts of autologous or allogeneic origin, were infected with wild-type or recombinant vaccinia virus, as indicated, by incubation with virus at a MOI of 20 for 1 hour in RPMI, then replacing the medium with complete RPMI and allowing the infection to proceed for 16 hours. The target cells (3 x 10^5) were then labeled at 37°C for 1 hour, by incubation in 0.5 mL of fetal calf serum (FCS) containing 100 mCi of Na^24CrO_4. Labeled cells were then washed and resuspended to 10^6 cells/mL in complete RPMI. For measurement of cytolytic activity, 1 x 10^5 ^51Cr-labeled target cells were incubated for 4 hours at 37°C with various numbers of effector cells, in a final volume of 200 μL per microtiter well, to give the described effector/target (E/T) cell ratios. After the incubation, the plates were centrifuged at 200g for 5 minutes and 100 μL of the supernatant was removed from each well for measuring the released radioactivity in a gamma counter. The percentage specific killing was calculated as follows: 100 x [(experimental ^51Cr release - spontaneous ^51Cr release)/total ^51Cr release - spontaneous ^51Cr release]). The release of ^51Cr by target cells cultured in medium alone was taken as spontaneous release, while ^51Cr release by target cells lysed with 1% Triton was measured as total release.

RESULTS

Secretion of soluble IL-2 receptors. Human T cells were previously found to disseminate fast into the internal organs of the transplanted mice. As early as 1 day after transplantation, human T cells were found not only in the peritoneum, but also in the liver, lungs, and spleen of the chimeric mice. The majority of human T cells in these mice have the phenotype of mature (CD4^+ or CD8^+) and memory (CD45RO^+) cells. Most of these T lymphocytes do not express IL-2 receptors (CD25^+), although about half of them are HLA-DR^+. Immunohistochemical staining of the different organs obtained from the hu-PBL-BALB mice showed that human B cells and T cells initially become attached to the peritoneum serosa as early as 2 days after transplantation, and then migrate into the mesentery under the serosa coating (Burakova et al, unpublished results). In agreement with the FACS data, human cells were found to migrate into secondary lymphoid organs: spleen, lymph nodes, Peyer's patches, and mucosal tissue.

Activation of T cells is associated with a dramatic increase in expression of the IL-2 receptor. In addition to the intact receptor found at the cell surface, activated T cells produce a truncated form of the receptor that is secreted as a soluble molecule (sIL-2R). Increased levels of this soluble form of the receptor for IL-2 have been found in various clinical situations associated with T-cell activation brought on by bacterial or viral infections, and in hematological disorders, autoimmune diseases, graft rejection, etc. We therefore measured the levels of human sIL-2R in the sera of hu-PBL-BALB chimeric mice to monitor the in vivo activation status of the human T cells. The level of sIL-2R was already detectable by day 4 posttransplant, reached a peak 1 week after transplant, and then declined to background level by 2 weeks posttransplant (Fig 1). The peak level (1.6 ng/mL) was comparable to the level found in humans during an immune system activation (data not shown).
mera-derived cells in response to stimulation with anti-CD3 monoclonal antibody OKT3 and/or human recombinant IL-2. Should the chimera-derived T cells be responsive to IL-2, but unresponsive to CD3 stimulation, this would suggest that these T cells were in an anergic state. Because human monocytes may be in insufficient numbers in our cell preparations,28 we used OKT3 monoclonal antibody immobilized on plastic at high density, rather than soluble antibody. Cells from hu-PBL-BALB chimeras proliferated to immobilized OKT3 when analyzed at various time points up to 1 month after the inoculation of human cells (Fig 2). In contrast, cells from control mice (not injected with human lymphocytes) did not proliferate above background levels in the presence of the monoclonal antibody and/or IL-2. Furthermore, the magnitude of the response was dependent on the concentration of human T cells in the analyzed sample. Peritoneal cells from hu-PBL-BALB mice contained a high percentage of human T cells, comparable to that of normal human PBL, and their proliferative response was also similar to that of normal human PBL. Exogenous IL-2 was not required for this response, and its addition did not lead to a marked further stimulation, suggesting that human T cells recovered from chimeric mice secrete enough IL-2 to support their own proliferation.

**Induction of activation markers on chimera-derived human T cells in response to anti-CD3 stimulation.** To study the activation of the chimera-derived human T cells in more depth, a sensitive three-color flow cytometric assay was used that identified specifically the human T lymphocytes recovered from hu-PBL-BALB mice by concomitant staining with CD45-fluorescein isothiocyanate (FITC) and CD4- or CD8-peridinin chlorophyll protein (PerCP), and simultaneously distinguished the activated T cells from the resting ones, by staining with phycoerythrin (PE)-conjugated anti-CD25 (IL-2 receptor) or HLA-DR antibodies. This assay allowed us to detect specifically the activation of human T cells, even in cell populations where they constitute a small percentage, such as the spleen of chimeric mice. In addition, the proportion of responding cells can be measured on the whole T-cell population (CD4/CD8+), as well as separately among the CD4 or CD8 subpopulations. Following a 3-day stimulation with OKT3 and IL-2, more than 92% of all human T cells (CD45/CD8+), recovered from the chimera mice at 12 days posttransplant, expressed the activation markers CD25 and HLA-DR, while the same stimulation induced in normal human PBL the expression of CD25 by only 75% and of HLA-DR by 16% of the T cells (data not shown). At another time point (21 days posttransplant), we monitored the induction of CD25 separately on CD4 and CD8 human cells (Fig 3). Stimulation with OKT3 monoclonal antibody alone induced the expression of CD25 on 84% and 80% of CD4 and CD8 cells, respectively, and these percentages were only slightly increased (to 90% of CD4 and 88% of CD8 cells) by adding exogenous IL-2 (10 U/mL). IL-2 alone induced the expression of CD25 on 20% of CD4 and 3% of CD8 cells (Fig 3). At the latest time point examined (33 days posttransplant), we sought to determine whether the human T cells that populate the spleens of the chimera mice are also responsive to anti-CD3 stimulation. Because of the limited number of cells available, only CD4+ T cells could be studied. Following a 3-day stimulation with OKT3 and IL-2, 70% of the human CD4 T cells expressed the activation marker CD25 (data not shown). Before the in vitro culture, only 3.5% of the CD4 cells were CD25+, while after a 3-day incubation in medium alone, 7% of the CD4 cells expressed this activation marker.

**Cytotoxic capability of human T cells from hu-PBL-BALB mice.** To study the xenoreactive cytotoxic activity of the human T cells in our hu-PBL-BALB mice, cells recovered from the peritoneal cavity of the BALB (H-2b) mice were tested for cytotoxic activity against two murine tumors, EL4 (H-2d) and P815 (H-2d), both of which are not congenic to BALB mice (H-2b). Figure 4 shows that the human cells recognize and kill specifically the target bearing the same major histocompatibility complex (MHC) molecules as the host (H-2b). The activity was strong, with a maximal killing of greater than 70% at an E/T ratio of 100 to 1, and was still detectable even at a 1 to 1 E/T ratio.

We next tested whether an allogeneic immune response could be induced in vivo in the mouse-human constructs. For this purpose, chimeric mice were immunized IP with irradiated allogeneic PBL, and the induction of antiallogeneic CTLs was tested 1 week after the immunization. To overcome the potential loss in the antigenic repertoire of the human T cells, mice were immunized with allogeneic cells on the day of the transplant. As shown in Fig 5A, the cells recovered from the chimeric mice (AB*) 1 week after immunization showed specific cytotoxicity against the immunizing allogeneic cells (from donor B), and not against third-party cells (donor C). The response was strong, with a maximal killing of greater than 70% at an E/T ratio of 100 to 1, and greater than 20% killing at a ratio of 0.8 to 1. This cytotoxic activity was induced by the immunization, as cells from nonimmunized mice (A) were not active (Fig 5A). It was also apparent that the activity was not mediated by the mouse cells from the chimeras, since it was directed specifically against the irradiated cells (donor B), and not against nonirra-
Rejection of tumor allograft by human cells from hu-PBL-BALB mice. To test the functional properties of the human cells in vivo, animals were transplanted subcutaneously with a human lymphoma, Raji, 1 week following transplantation of human PBL. One group of mice was immunized by IP injection of irradiated Raji cells, on the same day the PBL were transplanted. Peritoneal cells recovered 1 week later from the immunized mice displayed cytotoxic activity against Raji cells, indicating that the tumor was indeed immunogenic for the transplanted human PBL and that CTLs against it were generated in vivo (Fig 7). This cytotoxic activity was induced by immunization, since peritoneal cells from nonimmunized mice did not kill Raji cells. The chimeric mice that were immunized IP with irradiated Raji cells consequently rejected the subcutaneous tumor significantly faster (Fig 8) than the nonimmunized mice ($P = .016$) or control mice that did not receive human cells.

Induction of human CTLs in response to a viral infection. We used our mouse model to raise Nef-specific primary CTLs, to investigate whether a primary antiviral immune response could be induced in vivo. Chimeric mice were immunized IP with a live recombinant vaccinia-nef virus, and the induction of CTLs was tested 1 week after the immuniza-
FUNCTIONAL HUMAN T Cells IN CHIMERIC MICE

Stimulation

OKT3 + IL-2

OKT3

IL-2

Medium

CD4

Peritoneum 89.3 %

Peritoneum 83.9 %

Peritoneum 19.9 %

Peritoneum 2.6 %

CD25

CD8

CD25

CD8

CD25

CD8

CD25

CD8

Fig 3. Activation of human T cells recovered from BALB-human chimeras. Peritoneal cells were pooled from one group of chimeric mice (each group received human PBL from a single donor). The cells were recovered at 21 days posttransplant and were stimulated with immobilized OKT3 monoclonal antibody and/or IL-2, as described in the Methods. The induction of IL-2 receptors (CD25) was assessed by 3-color flow cytometry after a 72-hour incubation. A gate was set on all human cells (CD45+) and the induction of this activation antigen was measured separately on CD4+ and CD8+ cells, as indicated.

Fig 4. Antigenic cytotoxic activity of human cells from hu-PBL-BALB mice. The cytotoxic activity of human T cells recovered from the peritoneum of the chimeric mice was measured using the ⁴⁰K-release assay against mouse cell tumors P815 (H-2b) and EL4 (H-2b), as described in Materials and Methods.

DISCUSSION

Long-term studies of the hu-PBL-SCID model showed that human T cells from these mice become anergic and unresponsive to T cell receptor stimulation when tested late (30 to 90 days) after transplant, suggesting that chronic graft-versus-host disease, which is triggered by the reactivity of the human T cells against murine MHC products, could lead to this anergy.

One major difference between the classical hu-PBL-SCID model and ours is the kinetic of engraftment of human cells. In SCID mice, following IP injection of human PBL, the cells remain in the peritoneal cavity in small numbers for more than 30 days before expanding whereas in BALB recipients, the tempo of appearance of T cells is greatly enhanced and engraftment takes place within a few days posttransplant. The engraftment of human cells is likely improved by the lethal conditioning used before transplantation, and by increasing the inoculum of human cells (80 X 10⁶ per mouse). This strong and rapid engraftment enabled us to study the functionality of the human T cells earlier after transplantation of human PBL into the mice, compared with the long-
term studies of Tary et al (which were performed at 1 to 3 months posttransplant).

Other studies succeeded in obtaining engraftment and human T-cell responses in SCID mice within the first few weeks posttransplant through conditioning of recipient mice and by using a large inoculum of human cells, although this approach is limited by the lethal sensitivity of the SCID mice to the conditioning regimens reported to be effective. Thus, Alegre et al demonstrated that SCID mice engrafted with human splenocytes have functional human T cells, which are able to proliferate in response to immobilized OKT3 and to reject allogeneic human skin grafts. In this study, recipient SCID mice were conditioned by a 2-Gy irradiation before IP injection of a large number of human cells, and the experiments were performed early after engraftment of human cells into SCID mice (skin grafts at 11 to 14 days and proliferation assays at 3 weeks). In another study, Malkovska et al showed that human V-γ9N-62 cytotoxic T cells can be generated in vivo by immunization of hu-PBL-SCID mice with irradiated Daudi lymphoma, and that such mice are protected against subsequent IP challenge with the live lymphoma. These investigators also used an immunosuppressive regimen (IP injection of anti-asialo GM1 antibody) to deplete SCID mice of NK cells, used a large inoculum of human PBL (10⁶ per mouse), and performed the cytotoxic assays 1 to 2 weeks after human cell transplantation. However, these V-γ9N-62 cytotoxic T cells are not conventional allogeneic CTLs, because they recognize a homologous of the GroEL heat-shock proteins family, in a MHC-unrestricted manner reminiscent of a superantigen response. Our report is the first to demonstrate directly the generation of classical human antiallogeneic CTLs in mice, without any further stimulation in vitro. How-
FUNCTIONAL HUMAN T CELLS IN CHIMERIC MICE

Fig 8. Rejection of tumor allograft by hu-PBL-BALB mice. Chimeras were constructed as described and some of the mice were in addition immunized IP with 50 × 10⁶ irradiated (7,000 rad) Raji cells. One week later, all the mice were inoculated subcutaneously with Raji tumor cells. The tumor diameter was measured weekly and the results are expressed as mean tumor diameter ± SD.

Donor A

Donor B

Donor C

Donor D

Effector : Target ratio

Over, an antiallogeneic CTL response could be obtained only by immunizing the mice within the first week posttransplant, and not at later time points (Fig 6).

Immunization of mice with a tumor allograft is often used to generate allogeneic CTLs in vivo. We showed that human-ized chimeric mice immunized with a human lymphoma, Raji, develop human CTLs against these allogeneic cells (Fig 7). This correlated with an accelerated rejection of the subcutaneously transplanted tumor (Fig 8). Nevertheless, other nonspecific mechanisms may effect or contribute to this graft rejection, such as NK cells or various cytokines.⁷¹

The induction of antiviral CTL responses is believed to be an important component of a protective anti-HIV vaccine. HIV-specific CTLs can be readily detected in PBL obtained from seropositive individuals²³⁻²⁵ and, recently, a new method for evaluation of anti-HIV CTL responses allows detection of such CTLs in seronegative vaccine recipients for clinical trials of candidate HIV-1 vaccines.²⁶⁻²⁸ However, the protective effect of these CTLs against viral challenge cannot be examined in uninfected vaccine recipients. The hu-PBL-SCID mice were used to evaluate vaccine strategies for preventing HIV infection. SCID mice grafted with PBL from gp160-vaccinated donors were shown to resist HIV infection, and resistance correlated best with in vitro assays of cellular immunity.²⁹ In another study, adoptive transfer of a Nef-specific human CTL clone (generated from an HIV-seropositive individual) into hu-PBL-SCID mice protected them against HIV infection.³⁰ However, the hu-PBL-SCID mice in these studies were used only as recipients for the adoptive transfer of human cells that had been first exposed to antigen in the human donor. The ability to use a human-mouse chimeric model to generate Nef-specific human CTLs in vivo would represent a further advancement toward establishment of a model for vaccine testing from naive lympho-
cytes. Such a model could be used to test the induction of primary CTL responses by candidate and potentially hazardous HIV vaccines, as well as for a rapid evaluation of the protective effect of these vaccines. A previous study using a recombinant vaccinia virus in a murine model, to induce an in vivo CTL response to HIV-1 env, showed that CTL activity is detectable between days 7 and 28, with a peak on day 21 after immunization. To overcome the rapid disappearance of the human antigen presenting cells from our mice, as well as potential changes in the antigenic repertoire of the T cells, we attempted to detect nef-specific CTLs in our mice as early as 1 week after immunization. The cytotoxic activity of the freshly isolated cells was not MHC-restricted (Fig 9). Cells with NK-like activity, which have the ability to execute cytolysis in the absence of MHC restriction and which appear early in an antiviral response, may obscure specific CTL activity. Multiple cellular phenotypes and lineages, including those that express CD16, CD8, or CD3, with either an αβ or γδ T-cell receptor, may mediate such NK activity. Other studies demonstrated that only by deriving T-cell clones, the insert-specific CTLs induced by recombinant vaccinia vectors can be characterized in a definitive fashion. An AIDS vaccine clinical trial studied the induction of CTLs in volunteers immunized with a recombinant vaccinia carrying the env gene of HIV-1. The bulk, uncloned PBL, isolated from the vaccinees lysed almost equally well autologous cells infected with either the vac-env recombinant vector or with the parental vaccinia virus, and even uninfected cells. Only by deriving CTL clones a clear env-specific CTL activity could be demonstrated. Similar results had been obtained earlier in a primate system. Another study, concerned with the specificity of human anti-HIV CTLs, showed that the unfractionated PBLs from HIV-1-infected individuals lyse both autologous and allogeneic cells infected with recombinant vaccinia virus (vac-env) and also display a high background of cytotoxic activity against parental vaccinia infected cells. In contrast to this cytotoxic response detected with PBL, the cloned CTLs were MHC-restricted and env-specific. Because bulk uncultured virus-stimulated cells may contain NK-like cells that can mask viral antigen specificity of CTLs, we are currently working on the isolation of specific anti-Nef CTL clones.

The problems of the human-mouse chimeric models are due to the xenoreactivity of the human T cells toward murine cells, which leads the T cells to a state of continuous activation in vivo, and ultimately to exhaustion and anergy. Yet even before this anergy occurs, there is a selective expansion of the xenoreactive T cells, in detriment of the nonxenoreactive cells, which may restrict the antigenic repertoire of the human T cells. Human CD4+ clones that proliferate to H-2d stimulator cells can be isolated from hu-PBL-SCID mice. The hu-PBL-SCID constitute an in vivo culture system exhibiting several parameters associated with the xenogeneic transfer of human peripheral blood lymphocytes. Such a model could be used to test the induction of CTL responses against allogeneic lymphocytes and tumor cells, and in particular against a viral antigen in naive lymphocytes, might afford a useful preclinical model for the testing of immunosuppressive drugs or reactivity to vaccines, as well as for studying immune events occurring in transplantation or cancer. Since many human solid tumors and leukemias can also be transplanted in these mice, concomitantly with human PBL, they could provide a system for generating antitumor T cells and for evaluating the efficacy of antitumor immunizations.

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