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EFFECTS ON HUMAN AND NONHUMAN PRIMATE IMMUNE RESPONSE OF A NEW RAT ANTI-CD2 MONOCLONAL ANTIBODY[1]

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Background. **Nonhuman primate models are highly clinically relevant in transplantation. The development of immunosuppressive tools or a tolerogenic regimen for primate models therefore represents an important goal of transplantation immunological research. Hence, we have developed a rat monoclonal antibody (mAb) that recognizes the CD2 molecule (LO-CD2b) on both human and nonhuman primate cells.**

Methods. **The LO-CD2b mAb has been characterized by flow cytometry, E-rosetting inhibition, and Western blotting. In vitro inhibition of immune responses by LO-CD2b was assessed after both mitogenic and allogeneic stimulation in mixed lymphocyte reactions (MLR). Several LO-CD2b dose and time responses were tested. In vivo, peripheral and lymph node T-cell depletion was examined both by flow cytometry and immunohistology in 10 baboons that received intravenous injection of LO-CD2b at different doses and time courses. Xenosensitization (anti-rat) was assessed by ELISA. Renal allograft survival was followed in two baboons treated with iterative LO-CD2b injections.**

Results. **In vitro, LO-CD2b binds a lymphocyte antigenic determinant of 52 kDa that is recognized by other well-characterized anti-CD2 mAbs (T11, Leu5b). LO-CD2b recognized natural killer $CD2^+$ cells. Administration of 200 ng/ml LO-CD2b almost completely inhibited human and baboon mitogenic stimulation. Allogeneic baboon and human MLR were completely inhibited by the addition of LO-CD2b (at 312 ng/ml) on the day of the initiation of culture; when added after 1 or 2 days, LO-CD2b still provided a significant MLR inhibition (>50%). Incubation of LO-CD2b with baboon peripheral blood mononuclear cells produced very low cytokine levels (interferon-γ, tumor necrosis factor-α, interleukin 2). In secondary MLR, baboon peripheral blood mononuclear cells previously incubated with LO-CD2b were unable to respond to a second allogeneic stimulation but were able to react to mitogens. In vivo, within the first hour after LO-CD2b injection (at 0.15, 0.5, and 2 mg/kg), an 85–90% peripheral depletion of $CD2^+$ cells was observed. A partial T-cell depletion in inguinal lymph nodes was seen after 1 week. The mechanism of peripheral T-cell depletion could have been antibody-dependent cell cytotoxicity or opsonization but was complement independent. Iterative LO-CD2b injections (12 days at 0.35 mg/kg) slightly prolonged the renal allograft survival in two baboons.**

Conclusion. **LO-CD2b is a nonactivating rat anti-CD2 mAb able to strongly inhibit both mitogenic and allogeneic responses in human and nonhuman primates. In vivo, LO-CD2b provides a rapid peripheral T-cell depletion, which is reversible within days after the cessation of injections. This rat mAb represents a very important tool for in vivo experimental investigation in nonhuman primates because it similarly reacts against human T cells in vitro.**

Nonhuman primate models are highly clinically relevant in transplantation. The development of immunosuppressive tools or a tolerogenic regimen for primate models therefore represents an important goal of transplantation immunological research. In primates, there are successful reports of prolongation of vascularized allograft survival with immunosuppressive drugs or, more interestingly, of establishment of donor-specific immunological tolerance (*1-3*). In these experimental models, tolerance was often induced by the development of mixed hematopoietic chimerism and required nonmyeloablative total body irradiation in combination with donor bone marrow infusion and T cell-depleting immunosuppressive drugs. Tolerance has indeed been described as favored by a milieu with minimally reactive T cells (*4*). The use of total body irradiation in a clinical setting is, however, hazardous, and efforts must be carried out to establish immunosuppressive protocols using easily monitorable reagents that result in tolerance as previously reported in rodent studies (*5*). Recent reports showed, however, that tolerance to primarily vascularized allografts in primates might also be induced without irradiation and even without donor bone marrow infusion, by using a new anti-CD3-immunotoxin monoclonal antibody (mAb) (*4, 6, 7*). The profound and long-lasting T-cell depletion in both peripheral blood and lymph nodes may limit its clinical applicability, although another powerful mAb (Campath-1) has been successfully used in human clinical trials for bone marrow and renal allotransplantation (*8–11*).

Recently, a new mAb (LO-CD2a/BTI-322) has shown inter-

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esting immunosuppressive effects in human transplantation. BTI-322, which is an anti-human CD2 mAb, has demonstrated great efficiency in preventing acute cellular rejection in renal transplantation (*12*), in graft-versus-host disease, and in liver transplantation.[7] LO-CD2a, however, does not react with baboon or cynomolgus monkey T cells and therefore cannot be evaluated in tolerance experimental settings. We have therefore developed a new mAb (LO-CD2b) that shows similar properties to LO-CD2a but is active on both primate and human $CD2^+$ cells. LO-CD2b is a rat IgG2b mAb that recognizes both baboon and human $CD2^+$ T cells including $CD2^+$ natural killer (NK) cells. The purpose of this study was to determine whether LO-CD2b can inhibit human and nonhuman primate immune responses in vitro and nonhuman primate immune responses in vivo. The in vivo T-cell depletion was assessed in both peripheral blood and lymph nodes, and the prolongation of renal allograft survival was assessed in two animals. These preliminary results suggest that LO-CD2b is an interesting immunosuppressive agent that could be tested in immunosuppressive and/or tolerogenic protocols.

MATERIALS AND METHODS

In Vitro Studies

Production of LO-CD2b. The LO-CD2b hybridoma was produced by the fusion of splenocytes from a LOU/C rat immunized with human purified T cells and the nonsecreting rat fusion cell line IR983F (*13*). Purified human T lymphocytes (5×10^7) were injected intraperitoneally into the LOU/C rat two times at 3-week intervals. Fusion between the splenocytes from the immunized rat and the cell line was performed 4 days after the second sensitization. The mAb was produced in ascitic fluid and purified as previously described (*14*). Cell culture, cloning, isotyping, and cell staining were performed as previously reported (*15*).

Isolation of baboon and human peripheral blood mononuclear cells (PBMCs). Ten milliliters of heparinized blood was diluted in 25 ml of RPMI and the PBMCs were recovered after centrifugation across a density gradient on lymphocyte separation medium (LSM; International Medical, Brussels, Belgium). The isolated PBMCs were blended in enriched mixed lymphocyte reaction (MLR) medium: AIM medium (confidential composition of Gibco including gentamycin [10 μg/ml], streptomycin [50 μg/ml], human albumin 0.1%; Gibco, Verviers, Belgium) supplemented with 0. 1 mM minimum essential medium (Gibco), 1 mM sodium pyruvate (Gibco), 20 μM 2-mercaptoethanol, 2 mM L-glutamine (Gibco), 100 μg/ml penicillin (Gibco), 100 μg/ml streptomycin, and 2% decomplemented (56°C, 0.5 hr) baboon serum PBMCs were adjusted at 4×10^6 cells/ml and suspended in 100 μl of medium.

Inhibition of E-rosetting. After isolation with LSM, 10×10^6 cells/ml of baboon PBMCs were suspended in MLR medium. The cells were then incubated with either LO-CD2b or LO-DNP57 (IgG2b, isotype control) at a concentration of 10 μg/ml. After 30 min of incubation, the cells were washed two times with phosphate-buffered saline (PBS) (pH 7.2) containing 2% fetal calf serum and centrifuged (10 min, $400\times g$). The concentrated cells were then suspended in 1 ml of PBS and incubated for 1 hr with 2 ml of sheep red blood cells previously treated for 15 min at 37°C with a solution of 2-aminoethylisothiouronium bromide (pH 9). After washing, the supernatant was removed and the pellet suspended in 3 ml of PBS. Three milliliters of LSM was then added and the solution centrifuged for 20 min at $750\times g$. The pellet was recovered and washed in PBS containing 2% fetal calf serum. The rosette formation was assessed by observing an aliquot under the microscope. The number of rosettes was calculated for 100 cells.

Flow cytometry (FC). To study the possible competition between LO-CD2b and other well-characterized anti-CD2 mAbs such as T11 (number 660-23-89; Coulter, Hialeah, FL) or Leu5b (number 347-593; Becton Dickinson, Mountain View, CA), two experiments were performed. In the first experiment, baboon PBMCs were suspended simultaneously with 5 μg/ml of fluoresceinated Leu5b or T11 and serial dilutions of LO-CD2b (from 5 μg/ml to 18 ng/ml). After 30-min incubation and three washes, the percentage of cells labeled with fluorescein was assessed by FC. In the second experiment, the baboon PBMCs were suspended with serial dilutions of LO-CD2b (10 μg/ml to 19 ng/ml) and a fluoresceinated mouse anti-rat IgG2b (5 μg/ml). After 30-min incubation and three washes, 10 μg/ml of rhodamine-labeled T11 (number 660–28-68; Coulter) was added to each of the diluted LO-CD2b samples. The percentage of single- or double-positive cells was then assessed by FC.

Saturation test. Ten milliliters of heparinized baboon peripheral blood was diluted in 25 ml of RPMI, and the solution was centrifuged across a density gradient of LSM. The isolated PBMCs (7×10^6) cells/ml) were incubated for 30 min with 25 μl of naive baboon serum to saturate the Fc receptors. After two rinses, PBMCs were incubated with serial dilutions of LO-CD2b (from 80 g/ml to 0.3 ng/ml) for 30 min. The LO-CD2b coating was revealed using a peroxidase-labeled mouse anti-rat IgG2b (MARG-2b). The different samples were analyzed by FC.

Western blot. PBMCs were isolated from 40 ml of baboon heparinized blood by density gradient centrifugation on LSM. Isolated PBMCs (125×10^6 cells diluted in 100 μl of RPMI buffer) were suspended overnight with 200 μl of lysis buffer (0. 25 M Tris, pH 7.5; Triton 5%; NP-40 5%, dilution 10×) and with protease inhibitors (pepstatin 0.1 mg/ml; leupeptin 1 mg/ml; and phenylmethylsulfonyl fluoride 0.1 M) at a ratio of 1/100. After centrifugation, 25 μl of supernatant was placed on a Bis-Tris-HCl-buffered (pH 6.4) polyacrylamide gel (NuPAGE; NOVEX, San Diego, CA) under reducing conditions and transferred to a nitrocellulose membrane (NOVEX). After blocking with 1% skimmed milk (Merck, Darmstadt, Germany), the blot was incubated overnight with LO-CD2b diluted at 1/20,000 in PBS. After washing three times in PBS Tween buffer, the blot was incubated for 1 hr at 4°C with ^{125}I-anti-rat sheep red blood cells diluted 1/100 in PBS (50 μCi/0.6 ml; Amersham International, Buckinghamshire, UK). After incubation for 1 hr at 4°C, the blot was then washed with PBS Tween buffer and autoradiographed. A rat anti-CD2 and a mouse anti-rat IgG2b were used as controls.

Mitogenic cell stimulation. A total of 4×10^6 baboon and human PBMCs/ml were plated in 96-well microtiter plates (Falcon, Nunc Brand Products, Denmark) and either 20 μl of phytohemagglutinin (PHA; 80 μg/ml) or 20 μl of concanavalin A (ConA; 25 μg/ml) was added. LO-CD2b (200 ng/ml) was added 0, 24, and 48 hr after the beginning of the incubation. Immunocytoma IR863 (rat IgG2b) was used as control. Ninety-six hours after the initiation of the mitogenic stimulation, the plates were incubated for 4 hr with tritiated thymidine (1 mCi/ml; Amersham Life Sciences, Belgium), then harvested (Filtermate 196; Packard) and counted (Top count, microplate scintillator counter; Packard).

MLR. A total of 10^6 baboon or human PBMCs were suspended in 100 μl of AIM medium as responder cells and then mixed with a similar number of irradiated (25 Gy) PBMCs from another baboon or human, as stimulator cells. The culture was performed in triplicate in 96 U-well plates and incubated for 6 days at 37°C, 5% CO_2. After 6 days, the cells were pulsed with 25 μCi/well of tritiated thymidine for 5 hr, harvested, and counted. All results are expressed in raw counts per minute (cpm) as the mean of three wells. The LO-CD2b was added either at different concentrations (10 μg/ml to 610 pg/ml) from the beginning of the culture or at a constant concentration (5 or 0.5 μg/ml) on different days (day 0 to day 4) after the initiation of the culture. A rat anti-DNP antibody of IgG2b isotype (LO-DNP11) was used as negative control at the same concentration and the same

[7] Manuscript in preparation.

time. The percentage of MLR inhibition was obtained by the ratio of cpm from the wells with LO-CD2b to the cpm of the wells with the medium alone.

Secondary MLR. A total of 2×10^5 responder baboon lymphocytes were cultured with a similar amount of irradiated (25 Gy) lymphocytes from another baboon in the presence of LO-CD2b (100 ng/ml) for a 6-day primary culture. A rat anti-DNP antibody of IgG2b isotype (LO-DNP11) was used as negative control. After 6 days, the cells were harvested, washed, and maintained in culture for 3 additional days at 37°C without antibody. After 3 days, the cells were restimulated by the same irradiated stimulator cells (at a concentration of 2×10^5 cells/100 μl). Precultured cells were also stimulated with ConA (0.4/μg/ml) to compare an allogeneic and mitogenic stimulation. Three days after the initiation of this secondary culture, the plates were pulsed with [^{3}H]thymidine and harvested as described above.

Assessment of cytokine release (tumor necrosis factor [TNF]-α, interferon [IFN]-γ, and interleukin [IL]-2). Baboon PBMCs (1.5×10^6/ml) were incubated at 37°C with 5% CO_2 in enriched MLR medium for 12, 24, and 48 hr in the presence of LO-CD2b at different concentrations (1 μg/ml, 0.5 μg/ml, 125 ng/ml, and 250 ng/ml), rat immunocytoma IR863 (1 μg/ml), rat IgG2b isotype, 20 μl of lipopolysaccharide (LPS; 20 ng/ml), 20 μl of PHA (80 ng/ml), or 20 μl of ConA (25 μg/ml). After stimulation, the supernatants were collected and stored at −80°C until assayed for production of cytokines by using specific ELISA kits (Predicta; Genzyme, Cambridge, MA).

In Vivo Studies

Animals. Outbred baboons, male and female (*Papio hamadryas*), weighing 10–15 kg were used in this study. The animals were maintained in a restricted-access facility. All animals were negative for hepatitis and Simian T lymphotropic virus. The baboons received U.A.R. monkey 107 food ad libitum. Pairs of recipient and donor were matched for ABO blood groups. To select histoincompatible animals, one-way MLR was performed, and baboons that mounted a strong proliferative response (stimulation index at least >10) were selected. To assess the presence of anti-baboon antibodies, crossmatches were performed.

Transplantation. All animals were anesthetized with tiletamine/zolazepam (Zoletil 100; 6 mg/kg) for induction and enflurane (Ethrane 0.8%)/nitrous oxide (0.2 L/min) for maintenance. An endotracheal tube was used for continuous delivery of oxygen (0.5 L/min) during anesthesia. Buprenorphine (Temgesic) was given for 5 postoperative days with parenteral antibiotics (Clamoxyl). In the donor, the left kidney was harvested with a Carrel patch. A Dacron prosthesis was sutured on the aorta to maintain an appropriate aortic diameter and to maintain the baboon alive for future immunological testing. In recipients of allogeneic renal transplants, a bilateral nephrectomy was performed through a midline laparotomy. The kidney allograft was transplanted end-to-side to the aorta and vena cava in the iliac fossa by running sutures (Prolene 6-0; Ethicon, Johnson and Johnson, Somerville, NJ). The ureter was implanted into the bladder through a neocystostomy. A permanent catheter was positioned in the jugular vein and a jacket system (LOMIR, Canada) adapted to monitor several blood tests daily, such as plasma creatinine level, hematocrit, and lymphocyte counts without the need for anesthesia. Rejection was diagnosed when the renal function deteriorated >50% of the baseline creatinine level, and animals were euthanized when creatinine level persisted over 6 mg/dl for 2 days.

Experimental groups. In group 1, the baboons received a single injection of LO-CD2b mAb at different doses or multiple LO-CD2b injections at a similar dose but at different times of administration (3 or 12 days) (Table 1). LO-CD2b diluted in 100 ml of saline was injected over 1 hr through the jugular vein. These animals did not undergo a renal transplantation. In group 2, one nonimunosuppressed baboon received a renal allograft, as control for survival, and two animals received a 12-day course of LO-CD2b as well as a kidney renal allograft. Several additional experiments with one animal each were performed for comparison purposes: these three animals did not receive renal allografts and were treated as follows: animal 33 underwent a total body irradiation (2×1.5 Gy), animal 44 underwent a total body irradiation (2×1.5 Gy) as well as a 3-day regimen of LO-CD2b (2 mg/kg/day), and finally animal 39 received three doses (3×50 mg/kg) of antithymocyte globulin (ATG; Upjohn, Kalamazoo, MI).

Hematology analysis. Blood samples were taken daily and analyzed with a blood counter (MS9 vet; Melet Schloesing Lab, France). The total number of red blood cells, platelets, and leukocytes were obtained daily for all animals for at least 2 weeks.

Peripheral and lymph node lymphocyte monitoring. The phenotypes of peripheral lymphocyte subpopulations were monitored by FC analysis using a panel of labeled (fluorescein- or rhodamine-conjugated) mAbs including the following: anti-CD2 marker (T11, Coulter; and Leu-5b, Becton Dickinson), anti-CD4 (T4; Coulter), anti-CD20 (B1; Coulter), anti-CD3 (BioSource), and anti-CD8 (Leu-2a; Becton Dickinson). MARG-2b (mouse anti-rat IgG2b) and IR-863 (rat myeloma protein of IgG2b isotype) served as controls. Blood samples were taken at days 0, 3, 7, 10, 17, and 22 after treatment. The relative numbers of CD cell subpopulations were determined in each animal. The lymphocyte phenotype was similarly studied in the lymph node on days 0, 7, and 10. The lymph nodes were excised from the inguinal area. To estimate the T-cell depletion, the lymph node was crushed after excision, and the total cell count of lymphocytes was assessed per milligram of tissue. Cells were analyzed on a Becton Dickinson FACS analyzer.

TABLE 1. Experimental groups

Group	Baboon number	Treatment	Frequency & dosage	Renal allograft (+) survival days
Group 1 (single or multiple LO-CD2b intravenous injections)	11	LO-CD2b	1×0.45 mg/kg	—
	14	LO-CD2b	1×1 mg/kg	—
	32	LO-CD2b	1×2.5 mg/kg	—
	17	LO-CD2b	12×0.5 mg/kg	—
	35	LO-CD2b	3×0.5 mg/kg	—
	36	LO-CD2b	3×1 mg/kg	—
	42	LO-CD2b	3×2 mg/kg	—
Group 2 (renal allograft)	15	None	—	(+)10
	23	LO-CD2b	12×0.35 mg/kg	(+)20
	25	LO-CD2b	12×0.25 mg/kg	(+)15
Miscellaneous	39	ATG	3×50 mg/kg	
	33	Irradiation	2×1.5 Gy	
	44	Irradiation and LO-CD2b	2×1.5 Gy 3×2 mg/kg	

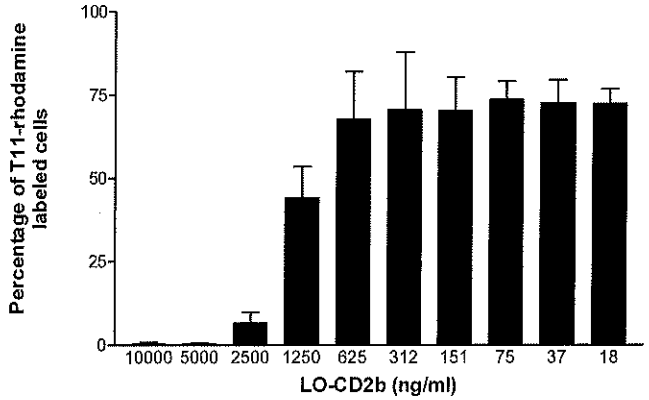

FIGURE 1. Serial dilutions of LO-CD2b (from 10 μg/ml to 18 ng/ml) were incubated with baboon PBMCs for 30 min and then with rhodamine-labeled T11 (anti-CD2) mAb. The percentage of rhodamine-labeled cells was assessed by FC and increased concomitantly with the decrease of the LO-CD2b dose, thereby demonstrating a specific competition for the CD2 molecule.

Anti-rat immunoglobulin response. After LO-CD2b treatment, plasma samples were assayed for the presence of monkey antibody to rat IgG2b by using an ELISA assay. Nitrocellulose (0.4 μm)-coated 96-well microtiter plates were incubated overnight with the rat IgG2b (LO-CD2b) mAb (10 μg/ml, 4°C) in borate buffer (pH 9.5). Plates were washed three times, incubated (1 hr at 37°C) with skimmed 5% milk in PBS, and washed three times in PBS 0.1% Tween. Microtiter plates were incubated with serial dilutions (1/200, 1/400, . . . 1/51,200) of monkey serum, and baboon Ig were detected by peroxidase-labeled rat anti-human IgM (LO-HM22) or IgG (LO-HG22). The plates were revealed using ortho-phenyldiamine (Sigma), 0.03% H_2O_2 (Merck) in citrate buffer (pH 5.5) solution. Optical density was measured at 492 nm using an optical reader (Labsystems, Multiskan RC, Finland).

LO-CD2b concentration. The serum concentration of LO-CD2b was assessed by ELISA. Briefly, the microtiter plates were coated overnight at 5°C with mouse anti-rat IgG2b, after saturation of nonspecific antigenic sites with skimmed milk. The plates were then incubated with serial dilutions of baboon serum. The rat Ig was detected by peroxidase-labeled mouse anti-rat kappa light chain mAb (MARK-1) and ortho-phenyldiamine development (Sigma). Purified rat IgG2b was used as standard. To evaluate the Ig serum concentration in treated monkeys, serial dilutions (1 μg/ml) of purified LO-CD2b were used, and the serum Ig concentration was measured after LOGIT transformation of standard and tested serum serial dilution curves.

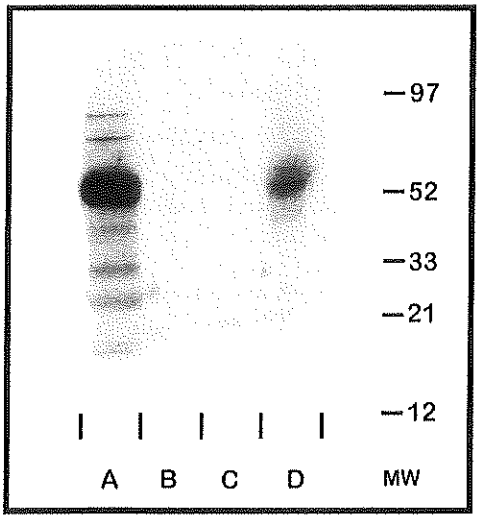

FIGURE 2. Western blot: ^{125}I anti-rat sheep Ig binding to (A) IgG2b rat mAb as a positive control; (B) IgG1b mouse Ig and (C) no reagent as negative controls; (D) supernatant of lysed PBMC+LO-CD2b. Molecular weights (MW) are shown on the right.

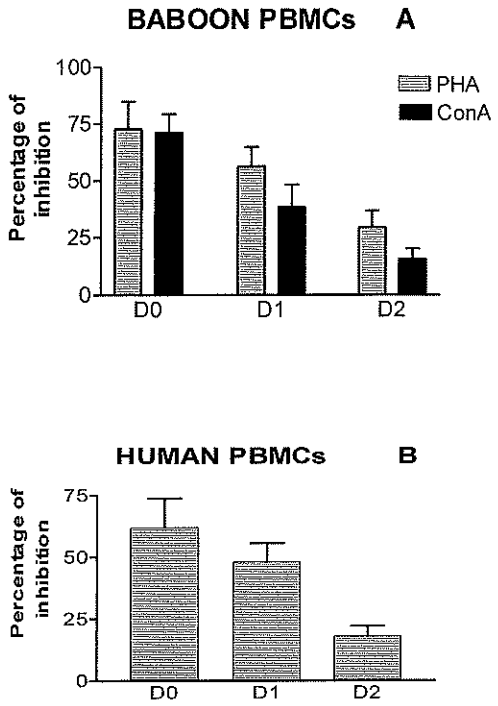

FIGURE 3. Effect of LO-CD2b on mitogenic stimulation. Baboon (A) or human (B) PBMCs were incubated with PHA (1.3 μg/ml) and/or ConA (0.4 μg/ml). LO-CD2b at a concentration of 200 ng/ml was added to the media on days 0, 1, and 2 after the beginning of the mitogenic stimulation. Results were obtained from three experiments (mean ± SEM) and are the percentage of inhibition of the proliferation after mitogenic stimulation. When LO-CD2b was added on the day of MLR initiation, the inhibition of proliferation induced by PHA reached 70% for baboon PBMCs and 60% for human PBMCs. Interestingly, even when added 1 day after the initiation of the MLR, there was still a 50% inhibition after the addition of LO-CD2b. Two days after the beginning of the MLR, LO-CD2b was able to inhibit 15–25% of the MLR with both baboon and human PBMCs.

Immunohistology. Biopsy specimens of inguinal lymph nodes were taken on days 0, 7, and 10 after the start of LO-CD2b treatment or total body irradiation, ATG treatment. The tissue was immediately placed in Bouin fixative, cut, and stained with hematoxylin and eosin for histological examination. For immunohistological studies, the lymph node tissue was washed in toluol, propanol, and ammoniacal acid, and the slides were suspended in 0.3% H_2O_2. After water and 50 mM Tris HCl washes, the nonspecific antigenic sites were saturated with normal goat serum (NGS) 1/10 and bovine serum albumin (1%) for 30 min. To evidence T, B, and macrophage cells, the tissue was incubated with rabbit anti-human CD3 (Dako), Clonab MB2 (all B cells; Biotest, Denville, NJ), and mouse anti-human CD68 (Dako) overnight at 4°C after the addition of Tris-NGS 1%. After washing in 0.05% Triton buffer, the slides were coated for 30 min with biotin-conjugated secondary antibodies (anti-mouse or anti-

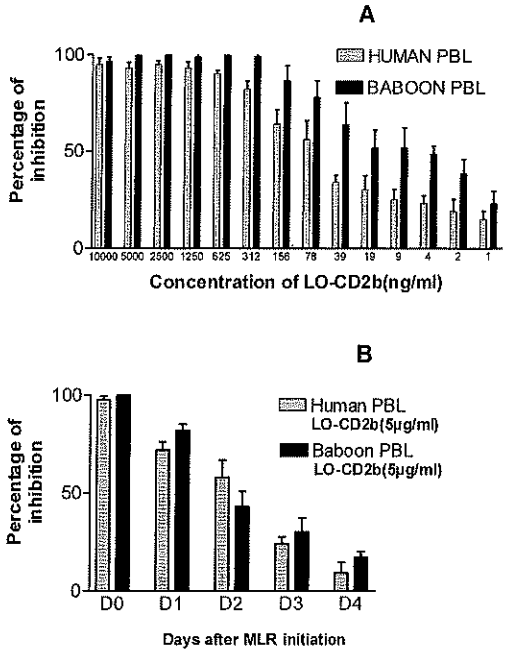

FIGURE 4. (A) Inhibition of allogeneic MLR by LO-CD2b. The mAb antibody was added at different concentrations (from 10 µg/ml to 1 ng/ml) at the beginning of an MLR between different baboons or humans. Between LO-CD2b concentrations of 10,000 and 312 ng/ml, the inhibition of the allogeneic baboon MLR was complete and reached 80–90% with human PBMCs. Between LO-CD2b concentrations of 156 and 1 ng/ml, the inhibition of the allogeneic MLR progressively decreased with both human and baboon PBMCs. (B) Course of MLR inhibition by LO-CD2b. The mAb (5 µg/ml) was added from the initiation of the MLR (D0) up to 4 days after the beginning of the MLR between two noncompatible monkeys or humans. Even when added 2 days after the initiation of the culture, LO-CD2b was still able to inhibit 50% of the MLR, and 15% when added 4 days after the beginning of the culture.

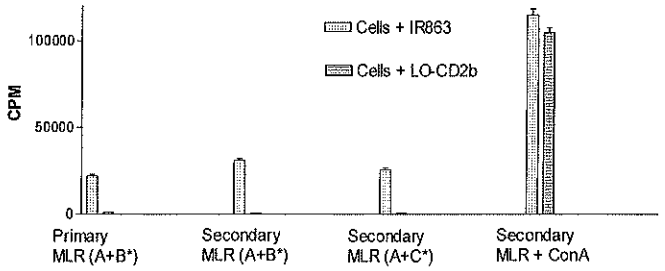

FIGURE 5. Secondary MLR. In primary allogeneic MLR the proliferation was high in the presence of IR863 (control antibody), whereas it was inhibited in the presence of LO-CD2b (100 ng/ml). In secondary MLR, the cells incubated previously with LO-CD2b were not able to proliferate when stimulated by two stimulator cells (B or C; B being the allogeneic stimulator used in the primary MLR), whereas cells incubated in primary MLR with the control antibody proliferated normally. One the other hand, cells previously incubated with LO-CD2b were stimulated by mitogen.

rabbit; Boehringer, Mannheim, Germany) in Tris-NGS 1% and skimmed milk (5%). Peroxidase-labeled streptavidin was deposited for 30 min after washing, and the peroxidase-labeled slides were revealed by diaminobenzidine, 30% hydrogen peroxide in water and in Tris-NGS 1% buffer. The reaction was stopped with water. The slides were then placed in hemalun, propanol, toluol, and xylene for 1 sec each.

Indium-111-oxine labeled leukocyte scintillography. Baboon PBMCs from 40 ml of heparinized peripheral blood were isolated, suspended in physiological water, and incubated for 15 min with 780 µCi (28,860 MBq) of ^{111}Indium-oxine (Amersham Healthcare) at 37°C. The complex is neutral, lipid soluble, and penetrates the cell membrane of the lymphocytes. Within the cell, the indium attaches to cytoplasmic components. The labeled cells were washed with 50% autologous plasma in ACD buffer and the free indium released by the cells. The pellet was resuspended in 4 ml of autologous plasma for injection. This suspension was injected in a male baboon of 40 kg (number 32). A blood sample was taken every 1–5 min during a 70-min experiment. Thoracic and abdominal scans were performed simultaneously. The scans were performed with a gamma-camera coupled to a computer with a medium energy collimator. LO-CD2b was injected at 2.5 mg/kg 20 min after the injection of the ^{111}Indium-labeled leukocytes.

Assessment of the classical and alternative pathway of complement (CH50 and AH50). Serum samples were collected before, one half hour after, and 1 hour after LO-CD2b administration and frozen at −80°C.

Classical pathway: 50 µl of a 1/10 dilution of each serum sample was prepared in ice-cold GVB++ buffer (0.2% gelatin; 3.5 mM veronal-buffered saline; 1 M HCl adjusted to pH 7.2; 0.01 M EDTA). Twenty-five microliters of a suspension of sheep erythrocytes sensitized with rabbit anti-sheep erythrocyte antibodies was added to each serum dilution sample to produce cellular antigen-antibody complexes. These complexes were incubated for 60 min at 37°C. A total of 1.2 ml of 0.15 M NaCl was added to each tube to stop the reaction, and after centrifugation for 5 min at 1250×*g*, the supernatants were analyzed on an OD 412 reader. CH50 U was defined as the dilution of serum that lyses 50% of the cells in the assay.

Alternative pathway: 100 µl of serial 1.5-fold dilutions of each serum sample in GVB/MgEDTA buffer was added with 100 µl of rabbit erythrocytes for 60 min at 37°C. A total of 1.2 ml of 0.15 M NaCl was added to each tube. The supernatant of each dilution was examined after 5 min of 1250×*g* centrifugation.

RESULTS

In Vitro Studies

LO-CD2b mAb binds to CD2 molecule. There was a partial competition between LO-CD2b and T11 or Leu5b for the same surface molecule. Figure 1 shows the mean of three experiments. This result suggested that LO-CD2b, Leu5b, and T11 recognize a closely associated epitope on CD2$^+$ cells.

In three experiments, the number of E-rosetting on 100 baboon lymphocytes was 6, 4, and 4 after incubation with LO-CD2b and 51, 59, and 56 after incubation with the control antibody (LO-DNP57), thereby providing a percentage of Rosetting inhibition for LO-CD2b of 88.2%, 93%, and 92%, respectively. LO-CD2b blocked E-rosetting at an average of 91±2% with a mean of 4.6 rosettes/100 cells for LO-CD2b-coated cells and 55.3 rosettes/100 cells for LO-DNP57.

In Figure 2, the Western blot showed that LO-CD2b is reactive with a lysed PBMC supernatant of a molecular weight of 52 kDa.

By FC, two saturation experiments showed that the mean

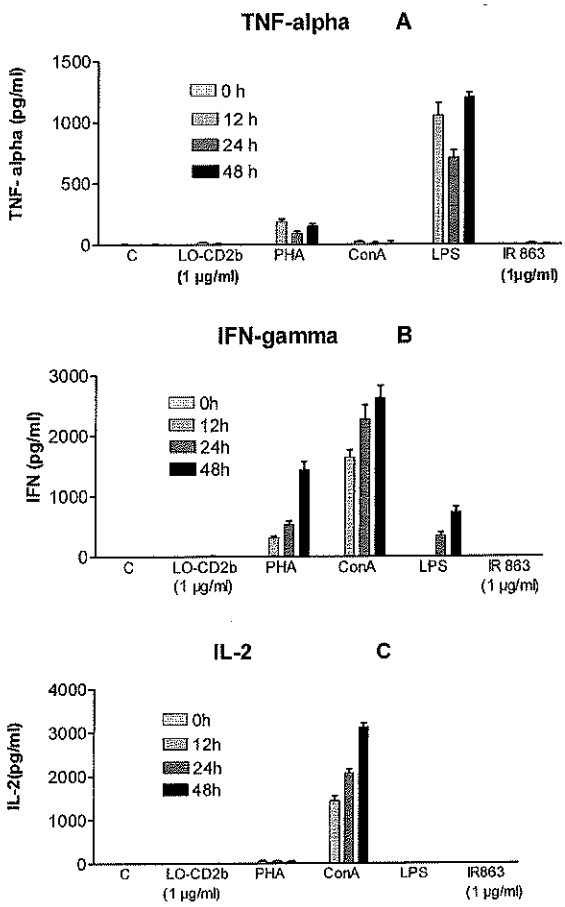

FIGURE 6. Effect of LO-CD2b and mitogenic agents on TNF-α (A), IFN-γ (B), and IL-2 (C) in vitro release. Baboon PBMCs were incubated with LO-CD2b (1 µg/ml), a rat immunocytoma IgG2b isotype IR863 (1 µg/ml), and several mitogenic agents (PHA, ConA, and LPS). Supernatants were taken at 0, 12, 24, and 48 hr to assess the cytokine production by ELISA (Genzyme). Results were expressed in pg/ml after two experiments. (A) The addition of PHA or LPS to the culture produced a significant production of TNF-α, whereas a dose of 1 µg/ml LO-CD2b has no effect. (B) The production in vitro of IFN-γ was significant after stimulation by PHA, ConA, and LPS, but no IFN-γ release was found after stimulation by LOCD2b. (C) The in vitro release of IL-2 was seen after stimulation by ConA but not after LO-CD2b stimulation.

fluorescence intensity increased from a concentration of 312 ng/ml until a plateau beginning at 2 µg/ml and above (data not shown).

Immune response. Mitogenic stimulation: When LO-CD2b was added at day 0, the PHA stimulation of baboon PBMCs was inhibited at 72.8±12.1%, and the ConA stimulation at 71.0±8.4%. LO-CD2b was still able to partially inhibit a mitogenic stimulation after the culture initiation: in fact, the addition of LO-CD2b at 1 or 2 days after culture initiation inhibited the PHA stimulation at 56±8.5% and 38.5±9.8%, respectively, and inhibited the ConA stimulation at 29.3±7.5% and 15.7±11.5%, respectively (Fig. 3A). LO-CD2b had a similar inhibitor effect on PHA-stimulated human PBMCs (Fig. 3B). In the presence of human PBMCs, the inhibition after PHA stimulation was 62.1% at day 0, 48% at day 1, and 18% at day 2.

Allogeneic stimulation—primary MLR: (1) Dose effects. When added at the day of initiation of culture, an LO-CD2B concentration more than 156 ng/ml almost completely (96.4±1.1%) inhibited a baboon allogeneic MLR. Low doses such as 4.8 and 9.7 ng/ml inhibited 50% of the proliferative response, and 1.2 ng/ml of LO-CD2b inhibited the MLR up to 22.9±14.1% (Fig. 4A). The same effect was observed in allogeneic human MLR but with a (10–20%) lower degree of inhibition. In comparison, no inhibition was obtained with a rat IgG2b isotype control (data not shown).

(2) Kinetic effects. Similarly to the inhibition obtained with mitogens, LO-CD2b was able to block an allogeneic stimulation even when added to human and baboon PBMC culture 1, 2, 3, and 4 days after initiation. Even 4 days after initiation, a 5 µg/ml concentration of LO-CD2b was able to inhibit the human and baboon allogeneic MLR up to 24.8% (Fig. 4B). When the cells were incubated with rat IgG2b isotype control (LO-DNP11) there was no inhibition (data not shown).

This inhibition effect was not the result of cell death because trypan blue exclusion (two repeated experiments) showed a stable percentage of cell death at days 1 and 3 after culture initiation for both baboon and human PBMCs. In the presence of LO-CD2b, the average of baboon cell death at days 1 and 3 was 11.2±1% and 17.1±3%, respectively, and in the presence of LO-DNP, 10.5±2.1% and 13.9±3.7%. Similarly for human PBMCs, the percentage of cell death at days 1 and 3 was 11±1.6% and 13.9±3.6% in the presence of

TABLE 2. Peripheral level of lymphocyte before and after LO-CD2b treatment (absolute number)

Baboons	D0[a]	1 hr	D1	D2	D3	D7	D10	D15	D20	D30
11 (1×0.45 mg/kg)	1700	700	0	ND	ND	ND	ND	ND	ND	ND
14 (1×1 mg/kg)	2900	200	0	2200	1400	3700	2700	3200	ND	ND
32 (1×2.5 mg/kg)	1770	400	830	1560	2041	2400	2100	1335	ND	3280
35 (3×0.5 mg/kg)	1640	340	680	600	1410	590	930	1140	930	1700
36 (3×1 mg/kg)	2220	370	570	760	350	330	4240	2350	1250	ND
42 (3×2 mg/kg)	2520	260	510	520	800	650	570	1670	2490	ND
17 (12×0.5 mg/kg)	4100	300	1000	800	900	600	900	500	1300	ND
23 (12×0.35 mg/kg)	3200	ND	700	400	800	0	0	0	0	ND
25 (12×0.25 mg/kg)	3100	ND	0	400	600	0	200	1100	ND	ND
15	3500	ND	2000	2800	2400	2300	3300	ND	ND	ND
39 ATG	1500	150	560	ND	680	1060	2900	2190	1400	ND
33 Irradiation	2030	1800	1060	320	300	270	220	370	630	2520
44 Irradiation and LO-CD2b	5440	1110	350	280	130	330	170	280	ND	ND

[a] Abbreviations used in tables: D, day; ND, not done.

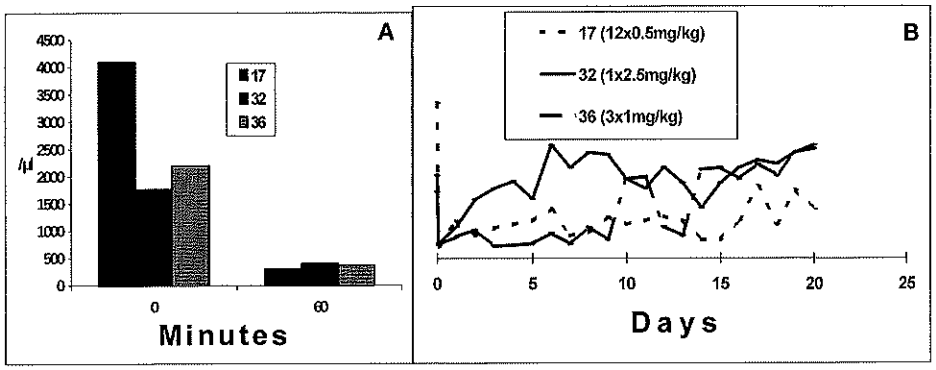

FIGURE 7. (A) Depletion of total lymphocytes in the peripheral blood during the first hour after LO-CD2b injection. The total count of peripheral lymphocytes dropped >80%. (B) Course of lymphocyte count in the peripheral blood after a single injection of LO-CD2b (baboon number 32), a 3-day regimen of LO-CD2b (baboon number 36), and a 12-day treatment of LO-CD2b (baboon number 17).

LO-CD2b and 9.5±3.2% and 16.2±3.9% in presence of LO-DNP, respectively.

Allogeneic stimulation—secondary MLR: After primary MLR, baboon responder PBMCs that had been previously incubated with LO-CD2b were unable to respond to a second stimulation with the same allogeneic stimulator cells, whereas these cells responded to mitogens. This result suggested the induction of hyporesponsiveness through T cell receptor interaction (Fig. 5).

Assessment of cytokine release after incubation with LO-CD2b (TNF-α, IFN-γ, and IL-2): Incubation of baboon PBMCs with different concentrations of LO-CD2b produced very low levels of TNF-α (<17.2 pg/ml) (Fig. 6A), IFN-γ (<126 pg/ml) (Fig. 6B), and IL-2 (<21.9 pg/ml) (Fig. 6C) after either 12, 24, or 48 hr. As positive control, the production of cytokines was significant when baboon PBMCs were stimulated by mitogens (PHA, ConA, and LPS). As negative control, PBMCs were incubated alone or with a rat IgG2b isotype control (IR863). This experiment showed that LO-CD2b does not activate baboon PBMCs and does not produce inflammatory cytokine release.

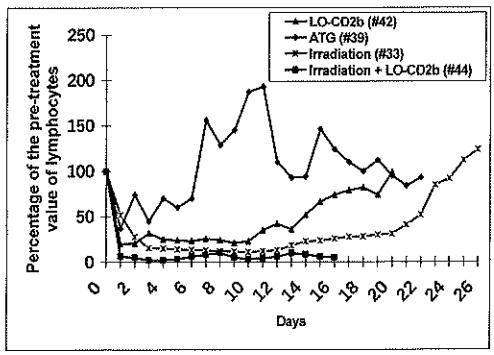

FIGURE 8. Course of total circulating lymphocytes count (expressed in % of the pretreatment value) in baboons treated with LO-CD2b (3×2 mg/kg), ATG (3×50 mg/kg), or total body irradiation (3 Gy) with or without LO-CD2b. LO-CD2b alone produced a rapid (within 1 hr) and strong (>80%) in vivo depletion during almost 2 weeks. In contrast, a 3-day regimen of ATG at 50 mg/kg did not provide a significant T-cell depletion. Total body irradiation alone or concomitant to a 3-day regimen of LO-CD2b provoked a more durable peripheral T-cell depletion.

In Vivo Studies

Peripheral lymphocyte depletion. The count of peripheral blood lymphocytes after different treatments is presented on Table 2. Independently of the LO-CD2b dose used, 1 hr after a single injection, 82.4±15.1% of the peripheral lymphocytes disappeared from the peripheral blood (Fig. 7A). After 24 hr, however, the recovery of the peripheral lymphocytes was dependent on the treatment used (Table 2): after a single injection, the lymphocytes reappeared in the peripheral blood 2–3 days after injection; after a 3-day course, the lymphocyte reappearance in the periphery occurred 1 week after the end of the treatment; and after a 12-day course, the peripheral blood depletion was maintained for at least 2 weeks (Fig. 7B). No obvious toxicity after single or iterative LO-CD2b administrations was evidenced in any baboons even at high doses such as 2 mg/kg.

In comparison to the LO-CD2b effect on peripheral blood cells, a total body irradiation of 2×1.5 Gy produced a slower but more profound depletion of the circulating peripheral T cells. Between 5 and 20 days after such an irradiation, only 25% of peripheral lymphocytes survived, and the preirradiation level was recovered after 3 weeks (Fig. 8). The addition of a 3-day regimen of LO-CD2b to a similar total body irradiation seemed to produce even a stronger peripheral T-cell depletion (the animal died on postirradiation day 16). In contrast, a 3-day regimen of ATG (50 mg/kg/day) did not provide a significant peripheral T-cell depletion, and 6 days after the third ATG injection the level of circulating lymphocytes rebounded above the pretreatment value. During the ATG treatment, the peripheral depletion never exceeded 50%.

Phenotype of peripheral lymphocytes after LO-CD2b treatment. As illustrated in Table 3, the absolute number of $CD2^+$, $CD20^+$, and $CD16^+/CD2^+$ cells are shown in one representative LO-CD2b treated (number 42) and one untreated animal (number 15). For all treated baboons, an average reduction of 92.9±5.1% of $CD2^+$ cells (81.3±7.3% to 5.6±3.9%) was observed between days 0 and 3. After 20 days, the percentage of $CD2^+$ cells was 36.3±16.8%. In contrast, the percentage of B lymphocytes ($CD20^+$ cells) increased

TABLE 3. Absolute number of $CD2^+$, $CD20^+$, and $CD16^+/CD2^+$ cells in peripheral blood before treatment (D0) and at days 3, 7, 10, 14, and 20

Baboons	CD	Blood (lymphocyte/μl)						Lymph node (10^3/mg)		
		D0	D3	D7	D10	D14	D20	D0	D7	D10
42	CD2	2142	0	78	142	726	1419	3000	280	2670
	CD20	231	313	280	269	524	366	176	129	180
	$CD16^+/CD2^+$	73	0	0	18	50	107	3.2	0	3
15	CD2	2800	ND	1472	2079	ND	ND	490	ND	572
	CD20	1050	ND	860	990	ND	ND	146	ND	193
	$CD16^+/CD2^+$	206	ND	57	99	ND	ND	2.5	ND	2.4

Absolute number of similarly coated cells in lymph node at D0, 7, and 10 in baboons 42 and 15.

from 19.9±13.2% to 45.0+21.7% during the same time period. The $CD2^+$ NK cells were also affected by the LO-CD2b treatment, because the percentage of $CD16^+/CD2^+$ cells dropped from 11.3±2.5% to 0.9±0.3% (a reduction of 92.0%) in the peripheral blood. These $CD16^+/CD2^+$ cells returned at the pretreatment level after 20 days.

Lymph node depletion: phenotype, absolute count, and immunohistology. Absolute number of cells in lymph node (Table 4): In LO-CD2b-treated baboons, the absolute number of mononuclear cells per milligram of tissue decreased between days 0 and 7 when an LO-CD2b dose of 1 or 2 mg/kg was used. The pretreatment level was, however, restored 10 days after the first injection. In comparison, one animal underwent a 3-day treatment with rabbit anti-T cell globulins (3×50 mg), but the absolute number of lymphocytes in the lymph node did not change significantly. In contrast, a total body irradiation (3 Gy) clearly provided a lymph node depletion that seemed more durable (more than day 10), and the addition of LO-CD2b to the total body irradiation was even more efficient in terms of lymph node depletion.

Phenotype: As illustrated in Table 3 for representative animal number 42, the absolute number of $CD2^+$ cells decreased between days 0 and 7 in the lymph node, but 10 days after the initiation of the treatment, the $CD2^+$ cells returned to pretreatment values. In contrast, the count of B lymphocytes slightly increased between days 0 and 7, to eventually return to the pretreatment level 10 days after treatment.

Lymph node immunohistology: Between days 0 and 10, a partial depletion of T cells was observed in the deep cortical area. At immunohistology, the $CD3^+$ cells seemed partially depleted at days 7 and 10 in comparison with day 0. In contrast, the B-cell population was increased, as assessed by MB2 staining, and more follicles were evidenced at days 7 and 10. In comparison, the baboon receiving a total body irradiation of 3 Gy showed a more significant T-cell depletion in inguinal lymph node as assessed by CD3 staining on days 7 and 10 after treatment (Fig. 9).

Free and coated LO-CD2b levels. The free LO-CD2b concentration in the sera of treated baboons was never high and did not exceed 2.5 μg/ml during LO-CD2b treatment. Such a concentration was only reached after high-dose injections (2 mg/kg). Despite frequent administrations (12 days), LO-CD2b was never found in excess after 5 or 10 days. To evidence the coated LO-CD2b mAb, PBMCs were incubated with fluoresceinated mouse anti-rat IgG2b mAb, and the percentage of positive cells was assessed by FC. From days 0 to 10, the percentage of coated cells varied between 2% and 26.5%. In the lymph nodes, LO-CD2b-coated T cells were not detected. These results are summarized in Table 5.

Immunization against LO-CD2b. Anti-rat antibodies were already detected 8 days after the first injection in all animals and became stronger after 10 days, whereas the sera before the treatment were uniformly negative (data not shown).

Mechanism of LO-CD2b T-cell depletion. Indium scintillography: As illustrated in Figure 10, the ^{111}Indium activity in the whole blood significantly decreased and there was no concomitant increase in the serum. This blood activity decrease was, however, correlated with a significant increase of the radioactivity in the liver and the spleen, thereby suggesting a sequestration of the PBMCs in the reticuloendothelial system of these two organs.

Complement dosage: The assessment of both classical and alternate pathways of the complement system did not vary after injection of LO-CD2b. This result suggested that the disappearance of the $CD2^+$ cells from the peripheral blood was not driven by a complement-dependent mechanism (Table 6).

Renal graft survival and histology. Without treatment, animal number 15 (control) died on postoperative day 10 in uremia. In comparison, animal number 25, which received a

TABLE 4. Total count of lymphocytes per milligram of tissue, recovered from inguinal lymph nodes at different days after the beginning of the treatment

Baboon number	D0	D7	D10	Treatment
35	460,000	670,000	610,000	LO-CD2b (3×0.5 mg/kg)
36	247,000	75,000	330,000	LO-CD2b (3×1 mg/kg)
42	3,200,000	1,000,000	3,000,000	LO-CD2b (3×2 mg/kg)
39	660,000	970,000	600,000	ATG (3×50 mg/kg)
33	780,000	360,000	420,000	Irradiation (2×3 Gy)
44	732,000	256,000	268,000	Irradiation (2×3 Gy and LO-CD2b (3×2 mg/kg)
15	860,000	ND	880,000	Control

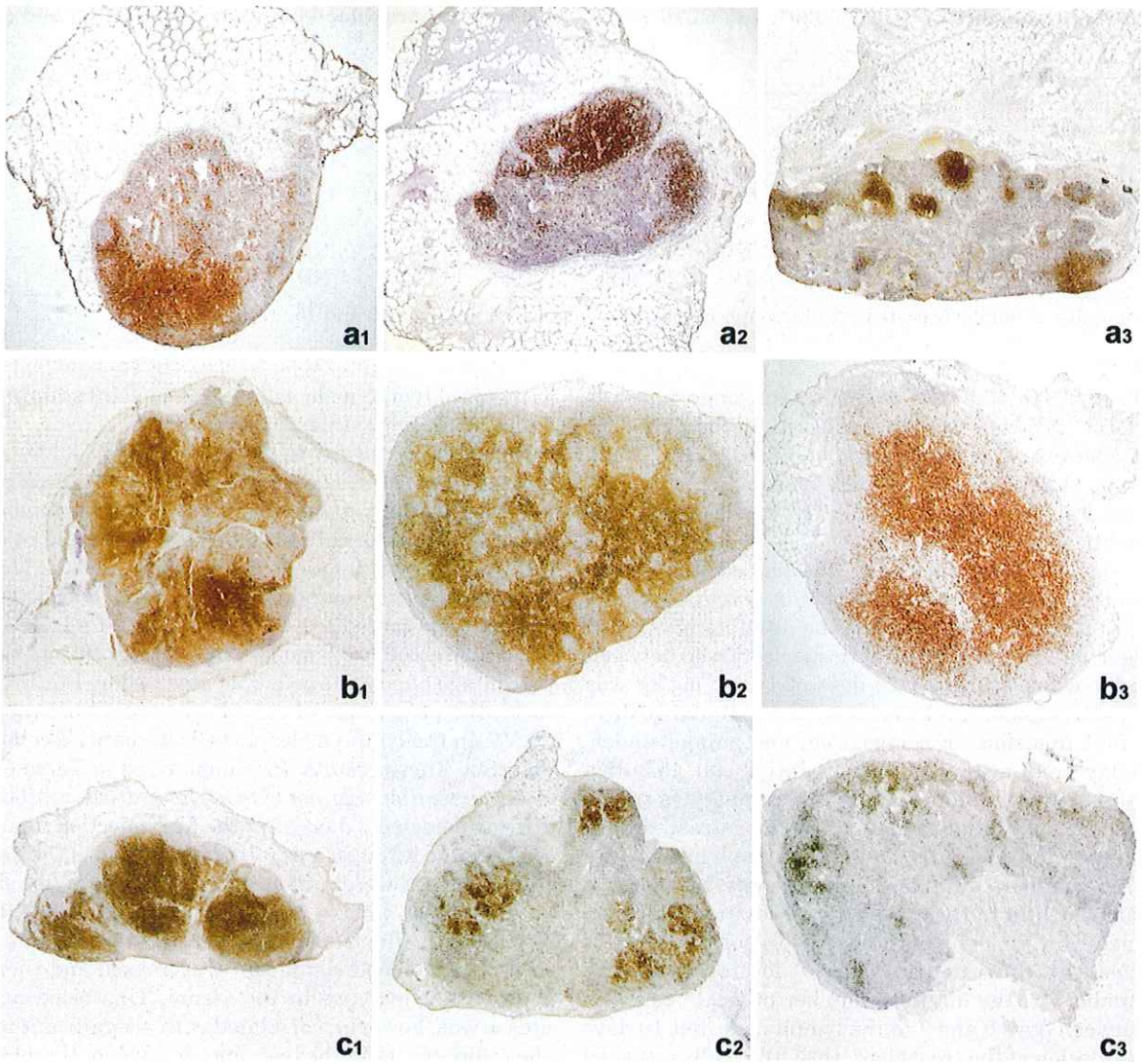

FIGURE 9. Immunohistology of baboon inguinal lymph nodes. The lymph nodes were colored by hematoxilin and eosin and labeled with peroxidized anti-CD3 mAb in order to evidence the T lymphocytes. In comparison with the pre-treatment level of T cells in the lymph node (a1), a TBI (3 Gy) provided a T cell depletion 7 days (a2) and 10 days (a3) after treatment. In comparison, a three day regimen of LO-CD2b at 2 mg/kg produced only a partial depletion of lymph node T cells 7 days (b2) after the beginning of the treatment (b1) and 10 days (b3) after treatment. The combination of both TBI and LO-CD2b treatment provided a similar T cell depletion than TBI alone on post-treatment day 7 (c2) and day 10 (c3). Pretreatment level (c1).

12-day regimen of LO-CD2b (0.25 mg/kg), rejected the renal allograft on postoperative day 15 (creatinine: 13.6 mg/ml). A second baboon (number 23) died on postoperative day 20 during anesthesia (for a surgical biopsy), whereas the renal function was normal (creatinine 0.8 mg/dl) as well as the histology (data not shown).

DISCUSSION

As evidenced by FC, E-rosetting, and Western blotting, LO-CD2b mAb interacts with the CD2 molecule on baboon PBMCs. LO-CD2b can completely inhibit mitogenic stimulation when added at the time of the initiation of the culture and even partially when added 3 days after the initiation. It inhibits the allogeneic MLR at subsaturating, nanogram concentrations, even when added as late as 4 days after the initiation of the culture. These results showed that LO-CD2b is able to block T cells at the initiation of the culture as well as activated T cells because the inhibitory effect is still important 1 or 2 days after culture initiation. In addition, LO-CD2b does not activate $CD2^+$ cells because no cytokine release was evidenced. These results differentiate this mAb from other anti-T cell mAbs such as OKT3, which eliminates but also activates T cells (*16*), and OKT11, which does not activate and does not inhibit T cells (*17*, *18*), or the blocking and nonactivating anti-Tac mAb (*19*, *20*). One of the most interesting characteristics of LO-CD2b is its ability to block both human and nonhuman primate allogeneic proliferative responses, thereby rendering it as an important experimental tool at both the preclinical and clinical levels. In addition, LO-CD2b seems to have similar in vitro and in vivo characteristics to LO-CD2a/BTI-322, which reverses renal rejection episodes (*12*), decreases the incidence of rejection when used

TABLE 5. Serum concentration (μg/ml) of free LO-CD2b (A) and percentage of LO-CD2b coated cells (revealed by fluoresceined mouse anti-rat IgG2b by FACs) (B)

A

Baboon number	Frequency and dosage of LO-CD2b	D0	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	D14	D15
11	1×0.45 mg/kg	0	0.5	0	0	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
14	1×1 mg/kg	0	1	0	0	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
32	1×2.5 mg/kg	0	1	0.2	0	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
17	12×0.5 mg/kg	0	0.1	0.3	0.3	0.5	1.6	0.3	0	0	0	0.1	0	0	0	0	0
35	3×0.5 mg/kg	0	0.1	0.2	0.2	0.5	0	0	0	0	0	0	ND	ND	ND	ND	ND
36	3×1 mg/kg	0	2.1	2.2	2.4	1.6	1	0.9	0.7	0	0	0	ND	ND	ND	ND	ND
42	3×2 mg/kg	0	2.4	2.3	2.0	1.4	1	1.2	1	0	0	0	ND	ND	ND	ND	ND
23	12×0.35 mg/kg	0	0.4	0.5	1.6	1.7	1	1.6	1.2	0.3	0	0	0	0	0	0	0
25	12×0.25 mg/kg	0	0	0.2	0.3	0.6	0.5	1	0.7	1	0	0	0	0	0	0	0

B

Baboon number	Frequency and dosage of LO-CD2b	D0	D3	D6	D9	D15
11	1×0.45 mg/kg	0.3	0.2	ND	ND	ND
14	1×1 mg/kg	0.5	0.6	ND	ND	ND
32	1×2.5 mg/kg	0.4	0.7	ND	ND	ND
17	12×0.5 mg/kg	0.2	18.0	0.1	0	ND
35	3×0.5 mg/kg	0.2	0.8	0.4	1	0.7
36	3×1 mg/kg	0.7	0.3	0.5	0.2	ND
42	3×2 mg/kg	0.4	0.2	0.3	0.2	ND
23	12×0.35 mg/kg	0.2	26.5	7.1	1	0.5
25	12×0.25 mg/kg	0.2	ND	0.6	0.2	0.4

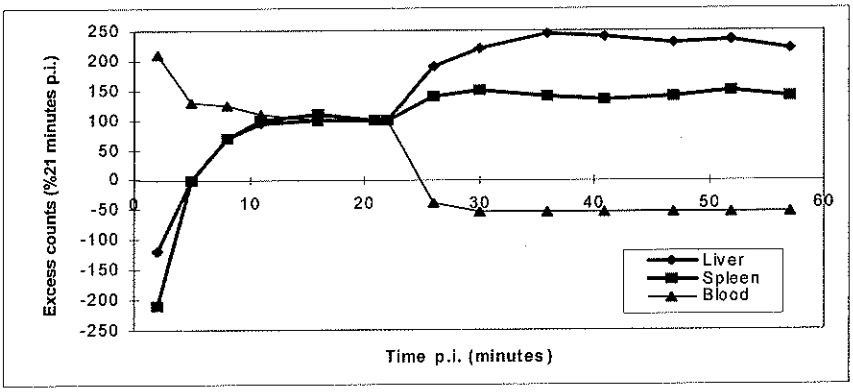

FIGURE 10. Indium scanning. The excess counts, expressed as the percentage of the radioactivity value at 21 min (time of LO-CD2b injection), represents the activity that was detected in liver, spleen, and peripheral blood. As illustrated on this figure, the excess count increased in both liver and spleen after LO-CD2b injection, whereas the count decreased in the peripheral blood. This result suggests a massive capture of the labeled lymphocytes in the reticuloendothelial system of these organs.

in prophylaxis (*17*), and is able to control cortico-steroid resistant graft-versus-host disease (*12, 17*). LO-CD2a has been humanized (MEDI-507; MedImmune, Gaithersburg, MD) and approved for use in clinical trials. LO-CD2b has the potential to become the experimental counterpart of LO-CD2a.

In vivo, LO-CD2b elicits a strong depletion of $CD2^+$ cells in the peripheral blood. Thirty minutes after a single-dose injection of LO-CD2b, the absolute count of lymphocytes dropped by more than 80%. Among the remaining cells in the peripheral blood lymphocytes, there were mainly $CD20^+$ cells, very few $CD2^+/CD4^+$ or $CD2^+/CD8^+$ T cells, and a small number of NK $CD2^-$ cells. The peripheral depletion was therefore important on both circulating T and NK $CD2^+$ cells but reversible several days after the treatment and directly dependent on the dose and duration used. In vivo, LO-CD2b seemed safe and seemed to deplete without activating lymphocytes or producing cytokine release. No side effects were evidenced after any dose or duration time period in vivo. The peripheral T-cell depletion obtained by LO-CD2b could be the result of several mechanisms: (i) by a complement-dependent way, but activation of neither classical nor alternate pathways of the complement were involved in our experiments; (ii) by a mechanism of antibody-dependent cell cytotoxicity; or (iii) by opsonization and destruction of the coated T cells in the reticuloendothelial system. Opsonization in fact seems to be involved as evidenced by the indium scanning data. The ligation of the T cell receptor by the CH2

TABLE 6. CH50 and AH50 values for two baboons injected i.v. with 2.5 mg/kg (baboon number 32) and 0.5 mg/kg (baboon number 35) LO-CD2b mAb. The serum samples were taken before, ½ hour after, and 1 hour after the injection.

	Bab 35		Bab 32	
	Classical pathway[a]	Alternative pathway[b]	Classical pathway[a]	Alternative pathway[b]
D0	43.2	27.2	74.5	46.1
D1/2h	46.8	26.7	78.0	44.0
D1h	31.5	26.3	76.1	45.1

No activation of the classical or alternate pathways of the complement was observed.
[a] Human normal range: 30–50 U.
[b] Human normal range: 28–144 U.

domain of the LO-CD2b antibody could result in opsonization and sequestration of LO-CD2b-coated cells by the reticuloendothelial system, which is particularly important in the liver and spleen. Coated T cells would be subsequently destroyed by macrophage phagocytosis or by other $FcRIII^+$ cells (*21, 22*).

The use of 3 Gy total body irradiation to effect peripheral T-cell depletion and which is used in protocols for inducing mixed chimerism (*1*) produced, in comparison, progressive but more profound and long-lasting T-cell depletion than LO-CD2b treatment alone. Similar results were obtained with the combination of total body irradiation and LO-CD2b. In contrast, ATG, which has been used in several protocols for inducing tolerance to primarily vascularized allografts in primates (*3, 23*), did not provide a strong and long-lasting T-cell depletion.

LO-CD2b elicits a strong depletion in the periphery but a moderate depletion in the lymph nodes at a dose higher than 1 mg/kg and during 3 consecutive days. After single or 3-day injections, there was a moderate depletion of T cells in the lymph nodes as assessed by the absolute number of lymphocytes per milligram of tissue. By FC, LO-CD2b effected a reduction in the percentage and in the mean fluorescence intensity of the $CD2^+$ cell population (also $CD4^+$ and $CD8^+$) between days 0 and 7. These results were confirmed by immunostaining experiments. This lymph node T-cell depletion was however less important than after a sublethal dose (3 Gy) of total body irradiation and than the lymph node depletion obtained with the combination of both agents. In contrast, ATG had no depletive effect on lymph nodes.

Peripheral depletion is not absolutely required for obtaining prolongation of primarily vascularized allografts. OKT4 (anti-CD4 mAb) in fact prolonged allograft survival in primates without $CD4^+$ T-cell depletion (*24*). On the other hand, several authors showed that when the number of peripheral blood T lymphocytes was reduced to below 10%, the incidence of rejection was reduced in treated renal allograft patients (*6, 7, 25*). To induce mixed chimerism, however, a peripheral T-cell depletion seems important at least transiently, and T-cell depletion at the level of the lymph node and thymus is also necessary from reports using rodents (*26, 27*). Transient but profound depletion of the recipient T-cell population seems to favor the establishment of tolerance. The replacement of total body irradiation by an easily controllable agent would of course be important to apply such tolerance protocols in humans, especially in children. Recently, the production of FN18-CRM9, composed of an anti-CD3 mAb and a binding site of diphtheria toxin, seems to be the first step in this direction, because this agent causes a strong and transient T-cell depletion in the peripheral blood (2–4 weeks) but also in the lymph nodes. This agent has shown remarkable success in primate renal allografts (*6*) and clearly demonstrated that tolerance could be obtained in primates without requiring total body irradiation or bone marrow infusion (*6, 7*). The use of such an immunosuppressive agent in humans will however require the evidence of its safety; among the main undesirable effects, the long-lasting peripheral T-cell depletion could prohibit its use in clinical trials. The preexistence of an immunization response against toxin diphtheria could also reduce the efficiency of this agent.

The lymph node depletion is pertinent for tolerance strategies, and peripheral T-cell depletion must persist for at least 1 or 2 weeks after completion of T cells (*28*). Relatively little data in primates and in humans have been published on the influence of antilymphocyte mAbs on lymphocytes in lymph nodes. The use of a humanized IgG1 anti-CD4 mAb results in a lower level of depletion in the lymph node than in the peripheral blood, whereas using the IgG4 isotype form of the humanized anti-CD4 mAb resulted in only a coating of $CD4^+$ cells and no depletion (*12, 24*).

Treatment with LO-CD2b as a single agent resulted in a modest but significant prolongation of allograft survival times. Although the untreated baboon died in uremia within 10 days after transplantation, the LO-CD2b regimen increased the graft survival up to 15 and 20 days. This latter animal died during anesthesia for surgical biopsy with a normal renal function and acceptable histology.

In conclusion, LO-CD2b is a nonactivating anti-CD2 mAb that inhibits allogeneic response and might induce antigen-specific unresponsiveness. In vivo, LO-CD2b produced a strong depletion of $CD2^+$ cells (T and NK cells) in the peripheral blood and a moderate depletion of $CD2^+$ T cells in lymph nodes with no apparent side effects. The depletion is, however, generally reversible within days after the end of the treatment and therefore monitorable. The mechanism of action of LO-CD2b could be either antibody-dependent cell cytotoxicity or opsonization in the reticuloendothelial system. LO-CD2b injections prolonged renal allograft survival in two baboons. These findings suggest that LO-CD2b is a potent immunosuppressive drug that should be tested in transplantation and in tolerance protocols. In addition, the fact that LO-CD2b seems to have the same characteristics as the humanized LO-CD2a (MEDI507), which already demonstrated efficacy in human clinical trials, renders LO-CD2b as a very important tool to study preclinical models of tolerance.

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