

ANTILYMPHOCYTIC ANTIBODIES AND MARROW TRANSPLANTATION

VI ABSENCE OF IMMUNOSUPPRESSION IN VIVO AFTER INJECTION OF MONOCLONAL ANTIBODIES BLOCKING GRAFT-VERSUS-HOST REACTIONS AND HUMORAL ANTIBODY FORMATION IN VITRO¹

STEFAN THIERFELDER,² GERTRUD HOFFMANN-FEZER, HANS RODT, ILIAS DOXIADIS,
MANFRED EULITZ, AND UDO KUMMER

Abteilung Immunologie, Institut für Hämatologie, GSF, Munich, Germany

The *in vivo* and *in vitro* effectiveness of several monoclonal antmouse T and B cell antibodies, of anti-Th-1 and of Ia^k serum, as well as of ATG were compared. The parameters were prolongation of skin graft survival, prevention of graft-versus-host disease (GVHD), antibody and primary and secondary plaque formation against sheep redblood cells (RBCs), and T cell depletion of lymphoid tissues. In general, *in vitro* effectiveness of the monoclonal antibodies exceeded their *in vivo* effectiveness. Skin graft survival was prolonged by ATG, but not by monoclonal anti-T, or anti-T plus anti-B antibody. GVHD was prevented by *in vitro* incubation of donor bone marrow with monoclonal anti-Th-1, but *in vivo* treatment of marrow donors was ineffective. Treatment with ATG was successful.

Anti Ia^k antibody blocked plaque formation by spleen cells incubated with sheep RBCs, but had no effect on secondary plaque formation when given *in vivo*. Neither was there any *in vivo* effect of anti-Ia^k or anti-Th-1 on antisheep RBC agglutinin formation. ATG was effective in both of these assays, although its cytotoxic and complement-fixing titer did not exceed that of anti-Th-1 or anti-Ia^k. Although anti-Th-1 was cleared more rapidly from the serum of mice expressing the corresponding Th-1 alloantigen, than from mice with the noncorresponding alloantigen and although anti-Th-1 was shown to bind to the T cell areas of the lymphoid tissue, it did not—unlike ATG—deplete these areas of T cells. Possible reasons for the difference in effectiveness of *in vitro* and *in vivo* application of these monoclonal antibodies are discussed.

A wealth of information exists about the *in vitro* immunosuppressive effect of monoclonal antilymphocyte antibodies. Information on their *in vivo* effectiveness is scanty. This is surprising since hybridoma-derived antibodies are already being produced for clinical purposes. The lack of data on *in vivo* immunosuppression in well-defined rodent systems is all the more unexpected because heterologous polyclonal antilymphocyte serum has quickly gained general acceptance for its immunosuppressive effect in rodents (1–3).

Reports on *in vivo* immunosuppressive monoclonal antibodies in rodents may be scarce because findings have been nega-

tive; for example, two monoclonal lymphocyte antibodies failed to prolong kidney graft survival in rats (4). By contrast, marrow treated *in vitro* with monoclonal antibodies against Th-1-positive cells caused no graft-versus-host disease (GVHD), or less severe reactions, after transfer into irradiated mice (5, 6).

The experiments reported here were designed to exclude the possibility that poor *in vivo* immunosuppression by monoclonal antibodies was due to peculiarities of certain murine hybridoma antibodies, such as lack of complement fixation, lack of *in vitro* cytotoxicity, or the presence of restricted lymphocyte antibody specificity. Several monoclonal antibodies to T and B lymphocytes were observed to be powerful immunosuppressants if permitted to bind to lymphocytes *in vitro*. They lost their immunosuppressive effect when injected into mice, although their complement and cytotoxic titers were similar to those of immunosuppressive rabbit ATG, and although they could be shown to bind specifically to their respective areas in the lymphoid tissue.

MATERIALS AND METHODS

Animals. 8–12-week old CBA, BALB/c, C57BL/6, (C57BL/6×CBA)F₁ (Th-1.2), and AKR/J (Th-1.1) mice were obtained from the Jackson Laboratory, Bar Harbor, ME, along with AKR/Cum (Th-1.2) mice from Cumberland View Farms, Clinton, TN. The two AKR sublines are antigenically similar with respect to markers Ly-1, Ly-2, Ly-3, TL, H-2, and G_{IX}, as well as with respect to gross cell surface antigen, but they differ for Thy-1 (7). Irradiated recipients of bone marrow or spleen cells were about 12 weeks old.

Irradiation. Mice were exposed in transparent, thermoplastic acrylic resin containers to 800–900 rads using ¹³⁷Cs (Gamma-cell, HWM-D-2000 [131 rads/min from opposing sources, each target distance 35 cm]) at 24 hr before transplantation of 2 × 10⁷ bone marrow and 5 × 10⁷ spleen cells.

Skin grafts. Tail skin grafts were placed on the lateral thoracic wall of allogeneic mice. They were covered with vaseline-impregnated gauze and adhesive tape. After 10 days the grafts were inspected every second day for signs of rejection.

Rabbit antimouse thymocyte globulin (ATG). New Zealand rabbits were immunized with 10⁸ AKR/J thymocytes on day 1, boosted on days 21, 22, and 23, and exsanguinated on day 28. The IgG fraction was prepared by means of ammonium sulfate precipitation and DEAE column chromatography (8).

Monoclonal antmouse Th-1 antibodies. In mice, thymus-derived lymphocytes express T antigen specificities in two allelic forms, Th-1.1 and Th-1.2 (9, 10), distributed reciprocally among various mouse strains. In rats only Th-1.1 has been

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² To whom reprint requests should be addressed, at Abteilung Immunologie, Institute für Hämatologie, GSF, 8 München 2, Landwehrstr. 61, Germany.

found so far (11, 12). For the skin grafting experiments we used supernates from 2 hybridomas established either against mouse Th-1.1 (IFH 1) or rat Th-1.1 thymocytes (MRC OX7).

IFH 1 was selected because of its complement-binding qualities. Both supernates were two (1:4000) titers above rabbit antimouse thymocyte globulin in microcytotoxic and micro-complement fixation tests when incubated with AKR/J thymocytes or spleen cell suspensions containing T cells. IFH 1 was derived from a hybrid cell line formed by polyethylene-glycol-mediated fusion of BALB/c spleen cells immunized with Th-1.1 thymocytes of AKR/J mice, and the myeloma cell line NSI/1 (according to the method of Köhler and Milstein [13]). Culture supernates revealed titers of 1:10⁶ when assayed by radioimmunoassay using ³H labeled antibodies (14).

IFH 1 is an IgM antibody. MRC OX7 is an IgG2A antibody (15). Its hybrid cell line (courtesy of Drs. Hunt and A. Williams, Oxford) was derived from BALB/c mice immunized with a glycoprotein isolated from rat thymocytes. MRC OX7 titers against Th-1.1 thymocytes are similar to those of IFH 1. Neither anti-Th-1.1 antibody reacts with Th-1.2 thymocytes of AKR/Cum mice. Monoclonal anti-Th-1.2 (NEI-001) was purchased from New England Nuclear, Boston, MA. It is an IgM antibody with titers similar to those of IFH 1, but with reciprocal specificity. Monoclonal anti-Ia^k, an IgG2A antibody (16) was derived from a hybrid cell line formed by fusion of spleen cells from BALB/c mice immunized with CBA spleen cells and the myeloma cell line NSI (according to the method of Köhler and Milstein [13]; courtesy of Dr. G. J. Hämmeling, DKFZ Heidelberg). It reacts strongly in the microcytotoxic test (around 1:2000), with about 90% of surface IgM-positive B cells, but not with T cells. The monoclonal antibodies were obtained as high-titered ascites fluids after injection of the respective cloned hybridomas into BALB/c mice that had previously received an intraperitoneal injection of mineral oil.

Lymphocytotoxic test. Lymphocytotoxicity was evaluated with the complement-dependent dye exclusions test (17). Non-cytotoxic rabbit serum was used as the complement source.

Complement fixation test. Quantitative complement fixation was adapted as a micromethod to determine membrane antigens (18), 2 units of guinea pig complement causing 50% hemolysis were added to 100 μ l of antibody at various dilutions and 100 μ l of lymphocytes (10⁷ cells/ml).

Immunohistochemistry. Cryostat sections, 7 μ m thick, were air dried and fixed in acetone for 5 min. The unlabeled antibody enzyme method originally described by Sternberger and Cuculis (19) and modified by Hoffmann-Fezer et al. (20) was carried out in three steps: (1) incubation with rabbit antimouse immunoglobulin (prepared against mouse IgG of MOPC 21); (2) incubation with sheep antirabbit immunoglobulin in excess; (3) incubation with peroxidase antiperoxidase complex. The biotin-avidin method (21) with biotinylated anti-Th-1.2 (NEI 010, New England Nuclear, Boston, MA) was used in the first incubation, and peroxidase-labeled avidin (Vector laboratories, Burlingame, CA) was used in the second. Each incubation lasted for 60 min, and was followed by washing in PBS. Peroxidase activity was revealed with aminoethylcarbazol (22). The sections were counterstained with hemalaune.

Hemolytic plaque assay. The immune response against sheep RBCs was tested 4 days after primary or secondary immunization using a method originally described by Jerne and Nordin (23). Direct hemolytic plaques (IgM response) were formed after adding a dilution of guinea pig complement. Further

incubation followed at 37°C for 1 hr. Indirect plaques (IgM + IgG response) were detected with antimouse Ig, as described by Dresser and Wortis (24). The Wilcoxin signed rank test was used for statistical analysis.

RESULTS

Skin graft rejection. Although treatment with ATG delayed rejection of H-2 incompatible skin grafts and secured the survival of non-H-2 grafts (Table 1, Nos. 1.2 and 2.2), during the observation time, antimouse Th-1.1 had no effect (Table 1, Nos. 1.3 and 2.3); antirat Th-1.1 (which crossreacts with mouse Th-1.1) was likewise ineffective. Since ATG also reacts with B lymphocytes, the T cell specificity of anti-Th-1.1 was complemented by simultaneous injections of anti-Ia^k, which is cytotoxic in vitro for the majority of B lymphocytes. This, however, did not prolong the survival of allogeneic skin on Th-1.1 Ia^k mice (Table 1, No. 2.4).

Graft-versus-host disease. Anti Th-1 was very effective for in vitro treatment of bone marrow to suppress GVHD in vivo. Bone marrow, which in mice has relatively few T lymphocytes, was supplemented by spleen cells that contain about 45% T lymphocytes and rapidly induce mortality within 3 weeks in H-2 incompatible mice. Acute and chronic GVHD subsequent to the transfer of 5×10^7 C57BL/6 cells was abolished in semiallogeneic H-2-incompatible F1 mice, and suppressed in 5 of 9 fully allogeneic CBA mice (Table 2, nos. 1.2, 1.4, and 3.2). The latter donor-recipient combination is particularly demanding, and requires the presence of guinea pig complement during the donor cell treatment with anti-Th-1.2. However, injection of anti-Th-1.1 into donor mice did not eliminate GVHD in (C57BL/6 $\times$ CBA)F₁ recipients (Table 2, No. 2.2). By contrast, the injection of ATG into donor mice abolished the GVHD-inducing potential of the spleen cells in secondary F₁ recipients (Table 2, No. 2.1).

Antibody formation against sheep red blood cells. Injection of anti-Th-1.1 into Th-1.1 mice reduced PFC from 615 to 197 (Table 3, Nos. 1.1 and 2.1), and injection of this substance into Th-1.2 mice reduced PFC from 628 to 333 (Table 3, Nos. 1.2 and 2.2). Although PFC reduction was significantly higher in the Th-1.1-positive cells ($P \leq 0.0012$) than in the Th-1.1-negative controls it was certainly less than the near-complete

TABLE 1. Survival of skin grafts on H-2-incompatible and non-H-2-incompatible mice after in vivo treatment with monoclonal antibodies against T or T and B lymphocytes, or treatment with ATG

Group	Serum treatment ^a	Strain ^b		Number of grafts surviving		
		Donor	Recipient	13 days	13-19 days	20-30 days
1.1	No serum	C57BL/6	AKR/J	18	4	0
1.2	ATG	C57BL/6	AKR/J	18	14	4
1.3	Anti-Th-1.1	C57BL/6	AKR/J	18	2	0
2.1	No serum	CBA	AKR/J	18	16	1
2.2	ATG	CBA	AKR/J	18	18	18
2.3	Anti-Th-1.1	CBA	AKR/J	16	14	2
2.4	Anti-Th-1.1 + Anti-Ia ^k	CBA	AKR/J	16	14	2

^a Amount: 0.25 ml/mouse/day from day -4 until day -1 before, and every third day after, skin grafting, until rejection. The antisera had comparable titers (Log₂ 10-12) in the microcytotoxic test.

^b Th-1 and MHC types: C57BL/6 (Th-1.2, H-2b), AKR/J (Th-1.1, H-2^k, Ia^k), CBA (Th-1.2, H-2^k, Ia^k), CBA, and AKR/J differ by non-H-2 antigens.

TABLE 2. Effects of in vivo or in vitro applied monoclonal anti-Th-1 or ATG on survival of graft-versus-host reactions in irradiated mice reconstituted with allogeneic spleen and bone marrow cells

Group	Serum treatment	Strain ^a		Survival		
		Donor	Recipients ^b	30 days	30-50 days	50 days
1.1	No serum	AKR/J	(C57BL/6×CBA)F ₁	10	2	0
1.2	Anti-Th-1.1 ^{c,d}	AKR/J	(C57BL/6×CBA)F ₁	10	10	10
2.1	ATG ^e	AKR/J	(C57BL/6×CBA)F ₁	12	12	12
2.2	Anti-Th-1.1 ^f	AKR/J	(C57BL/6×CBA)F ₁	9	0	0
3.1	No serum	C57BL/6	CBA	12	0	0
3.2	Anti-Th-1.2 ^e	C57BL/6	CBA	9	7	5
1.3	Anti-Th-1.1 ^c	AKR/Cum	(C57BL/6×CBA)F ₁	6	3	0
1.4	Anti-Th-1.2 ^c	AKR/Cum	(C57BL/6×CBA)F ₁	8	8	8

^a Th-1 and H-2 types: AKR/J (Th-1.1, H-2^k), AKR/Cum (Th-1.2, H-2^k), C57BL/6 (Th-1.2, H-2^b), CBA (Th-1.2, H-2^k).

^b Conditioned with 850 rads¹³⁷ Cs, 131 rads/min, 24 hr before injection of 5×10^7 spleen and 2×10^7 bone marrow cells.

^c Serum treatment in vitro of donor cells at a dilution of 1:20.

^d Incubation of donor cells for 30 min at 4 C.

^e Incubation of donor cells together with C' (guinea pig) 60 min at 37 C.

^f Serum treatment in vivo of spleen cell donors with 0.25 ml/mouse/day from day -4 until day -1 before transplantation.

TABLE 3. Immune response to sheep red blood cells in mice pretreated with monoclonal anti-Th-1.1., anti-Ia^k, or ATG in vivo

Group	Serum treatment ^a	Strain	Plaques per 10 ⁶ spleen cells ^b	Agglutination (Log ₂ titer) ^{b,c}
Primary immunization ^d				
1.1	—	AKR/J	615 ± 58	9
1.2	—	AKR/Cum	628 ± 46	10.7 ± 0.5
2.1	Anti-Th-1.1	AKR/J	197 ± 81	8.25 ± 0.5
2.2	—	AKR/Cum	333 ± 60	11
2.3	Anti-Ia ^k	AKR/J	6 ± 2	6.2 ± 0.9
2.4	ATG	—	2 ± 0.8	0
Secondary Immunization ^e				
3.1	—	AKR/J	5.3 ± 25 ^f	9.2 ± 0.5
3.2	—	—	153 ± 61 ^g	9.2 ± 0.5
4.1	anti-Ia ^k	—	202 ± 31 ^f	8.2 ± 1
4.2	—	—	173 ± 35 ^g	—
5.1	ATG	—	7 ± 3 ^f	—
5.2	—	—	5 ± 2 ^g	4

^a 0.25 ml/day/mouse from day -4 until day -1 before injection of 10⁸ sheep RBCs.

^b Mean and SD from 4 mice per group.

^c Nine days after immunization.

^d Four days after primary immunization

^e Four days after secondary immunization.

^f Direct plaque-forming cells.

^g Indirect plaque-forming cells.

inhibition of PFC in mice injected with anti-Ia^k or ATG (Table 3, Nos. 2.3 and 2.4). However, in contrast to ATG (Table 3, Nos. 5.1 and 5.2), anti-Ia^k did not suppress the secondary PFC immune response (Table 3, Nos. 4.1 and 4.2).

Injection of anti-Th-1.1 into Th-1.1 or Th-1.2 mice did not substantially affect the titers of agglutinating antibodies against sheep RBCs (Table 3, Nos. 1.1, 2.1, 1.2, and 2.2). Interestingly, anti-Ia^k, which markedly inhibited PFC formation, merely reduced the titer of agglutinating antibody from 9 to 6.2 Log₂ after primary immunization (Table 3, Nos. 1.1 and 2.3) whereas ATG virtually abolished hemagglutination (Table 3, No. 2.4). Furthermore, again in contrast to ATG (Table 3, No. 5.2), anti-Ia^k failed to reduce PFC and titers of hemagglutinins (Table 3, Nos. 3.2 and 4.2) after secondary immunization.

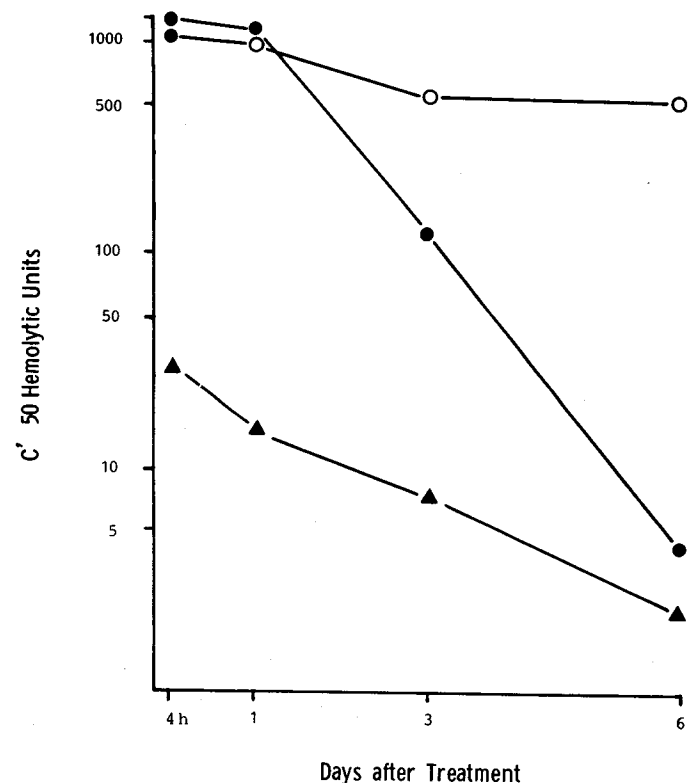


FIGURE 1. Antibody clearance of mice pretreated with anti-Th-1.1 or ATG. AKR/J (Th-1.1) mice were injected with anti-Th-1.1 (●—●) or ATG ▲—▲). AKR/Cum (Th-1.2) (○—○) were injected with anti-Th-1.1. Quantitative complement fixation of serum from injected mice was tested in the presence of AKR/J (Th-1.1) thymocytes.

Antibody Clearance. 0.25 ml anti-Th-1.1 (MRC OX7) or ATG was injected daily for 4 days into AKR/J mice. The antibody preparation had been adjusted to the same complement-fixing activity in the presence of AKR thymocytes. At 1, 3, and 6 days after antibody treatment, the complement-fixing activity of the serum of the injected mice was tested with AKR/J thymocytes. As a negative control, anti-Th-1.1 was also injected into Th-1.2 AKR/Cum mice under otherwise identical conditions. As can be seen in Figure 1, ATG disappeared quickly. Already 4 hr after injection of ATG the complement-fixing activity

against AKR/J thymocytes was only about 1/400 of that measured in the serum of Th-1.1 AKR/J mice injected with anti-Th-1.1. Complement fixing anti-Th-1.1 activity remained high after injection of anti-Th-1.1 in the serum of Th-1.2 AKR/Cum mice, but specific immune elimination of anti-Th-1.1 in Th-1.1 AKR/J had occurred by day 6 after termination of antibody treatment.

Fate of T cells in lymphoid tissue after injection of anti-Th-1. Th-1.1 and Th-1.2 mice received 0.25 ml of ATG, anti-Th-1.1, or anti-Th-1.2 daily for 4 days. Thereafter paracortical lymph node and periarteriolar splenic T cell areas were evaluated using an immunohistochemical (PAP) staining method. The splenic periarteriolar T cell zone of Th-1.2 mice treated with anti-Th-1.2 was full of lymphocytes stained with anti-Th-1.2, although ATG-treated mice showed cell depletion in the T cell

areas (Fig. 2). Injection of monoclonal anti-Th-1 together with anti-Ia^k did not enhance cell depletion of lymphoid tissue, although specific attachment of murine antibodies to the lymphoid cells could be demonstrated in spleen sections using rabbit antimouse globulin.

DISCUSSION

The reported data seem to be contradictory: monoclonal anti-Th-1 antibodies blocked splenic lymphocytes during in vitro incubation and thereby prevented GVHD after transfer of these cells into conditioned mice. The same antibodies did not reduce the GVHD-inducing potential of T cells when injected into mice in which the spleen cells were subsequently transferred to irradiated secondary recipients (Table 2, No. 2.2).

In contrast, rabbit ATG injected into spleen cell donors

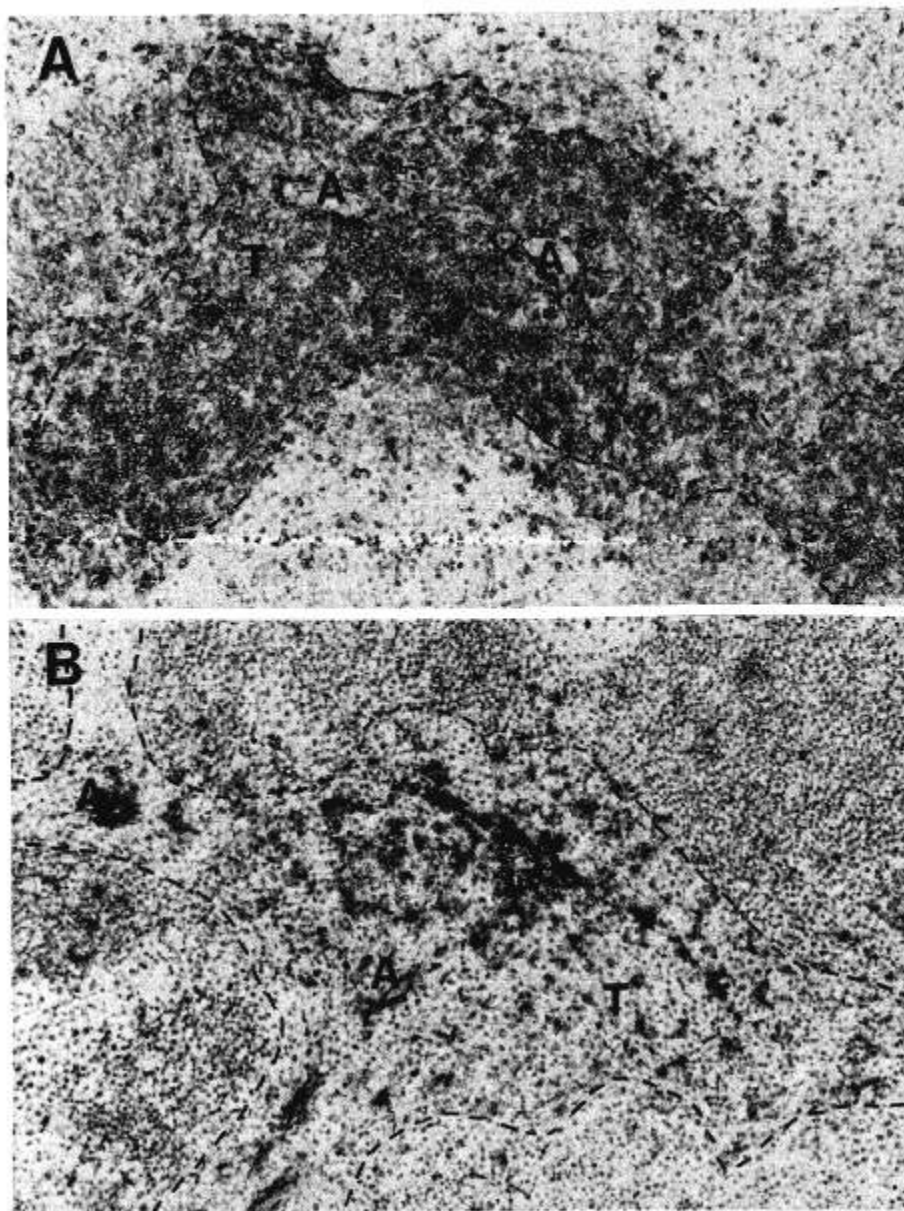


FIGURE 2. Immunohistochemical localization of T lymphocytes in Th-1.2 mice pretreated with anti-Th-1.2 (a) or with ATG (b). Dotted line = border between T and B cell area of the splenic white pulp; A = arteriole; T = periarteriolar T cell zone. Frozen sections were first incubated with biotinylated anti-Th-1.2 (monoclonal) and then, after washing, with horseradish-peroxidase Avidin-D. Note massive depletion of T cells with ATG.

abolished their GVHD-inducing potential in secondary H-2-incompatible F_1 recipients. Suppression of acute and chronic GVHD (25, 26) and prolongation of skin graft survival in rodents after ATG have long been known (1, 2) and have served as positive controls of immunosuppression in this investigation.

The failure of monoclonal anti-Th-1 antibody to delay GVHD and skin graft survival when injected directly into mice is not explained by low cytotoxicity or failure to fix complement. We used monoclonal IgM and IgG antibodies with cytotoxic and complement-fixing titers that were 2 dilutions higher than those of rabbit ATG. It is true that these *in vitro* tests use rabbit or guinea pig rather than mouse complement. It is, however, unlikely that our monoclonal antibodies, which fixed more guinea pig complement *in vitro* than ATG, were less cytolytic in mice. Both types of antibodies had been heat-inactivated prior to injection into mice in order to make sure that any lymphocytic effect depended on endogenous mouse complement. Monoclonal anti-Th-1.1 cleared more rapidly from Th-1.1-antigen-expressing mice than from Th-1.2-antigen-expressing mice, which agrees with reports (27) of immune clearance of ^{125}I -labeled anti-Th-1.1 from the blood of AKR/J mice. Instead of using isotope-labeled anti-Th-1, we recorded the clearance of unconjugated anti-Th-1.1 serologically; it was done by means of quantitative complement fixation using mice pretreated with anti-Th-1.1 for 4 days. The resulting clearance showed that over 95 % of the ATG had already disappeared 4 hr after injection, whereas most of the anti-Th-1.1 activity was still in the serum of the mice 24 hr later (Fig. 1). The slower disappearance of anti-Th-1 may reflect the slower consumption of the antibody by its target cells. This interpretation would be compatible with our immunohistochemical findings: anti-Th-1 was localized immunohistochemically on lymphocytes of the thymus-dependent interfollicular areas of lymph nodes, and in the periarteriolar T cell zone of the splenic white pulp. Although anti-Th-1 evidently reached its targets, depletion was not as severe as with ATG. Immunohistochemical methods had not so far been employed in the study of immunosuppression. Their advantage is that they localize lymphocytes according to their surface markers (20).

Rabbit ATG represents a mixture of antibodies of various specificities, including those against B lymphocytes and macrophages; we therefore injected cytotoxic monoclonal anti- Ia^k together with monoclonal anti-Th-1. Anti- Ia^k reacts with most B lymphocytes and macrophages. This, however, affected skin graft survival as little as the anti-Th-1 alone. Anti- Ia^k (in contrast to anti-Th-1.1) blocked the humoral plaque-forming response against sheep RBCs after primary immunization. This was expected from previous reports of polyclonal anti- Ia^k (28, 29) against xenogeneic RBCs and haptens in microculture systems. In addition, we observed that injection of anti- Ia^k into mice had only borderline *in vivo* effects on the formation of hemagglutinins against sheep RBCs. Our experiments document the weak *in vivo* effect of monoclonal antibodies on antibody formation, as compared with that of ATG. They also point to a failure of these antibodies to interfere *in vivo* with T-B cell cooperation, in contrast with ATG. This lack of interference does not seem to be due to a poor binding of anti-Th-1 antibodies to their target cells, because preliminary affinity studies did not reveal binding affinity of our anti-Th-1 that was lower than that of ATG (Kummer et al., unpublished data).

ATG, unlike our murine monoclonal antibodies, is xeno-

genic for mice. Xenogenicity, however, should not be a major reason why ATG is immunosuppressive in mice, because rabbit ATG prolongs skin graft survival just as well, if not better, in rodents made tolerant to rabbit Ig by low-zone tolerance (30-32).

Our investigations suggest that monoclonal murine antibodies against certain T-lymphocytic or B lymphocytic alloantigens, or both, reach their targets, but fail to deplete them as quickly as rabbit ATG does. In consequence, they fail to suppress *in vivo* cellular or humoral immune responses. Conceivably, lymphocytes survive certain antibody attacks by shedding their antigens. Anti-Th-1 may be shed more easily than other antibodies, such as anti-Lyt-1.1, which has very recently been reported to delay skin graft survival (33). Conceivably, more group-specific or species-specific sites, or different ones, need to be covered by antibodies before they perish.

Although our monoclonal anti-Th-1 antibodies were ineffective following *in vivo* application, their ability to suppress GVHD if applied *in vitro*, prior to transplantation, may well prove useful. This suppression resembles the effect of polyclonal mouse anti-T-cell serum (34, 35), and also that of specifically absorbed rabbit anti-T-cell serum (8, 36-38), which suppresses GVHD in dogs (39) and is presently undergoing clinical investigation (5).

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