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SUPPRESSORS OF THE GRAFT VERSUS GRAFT REACTION IN TOLERANCE TO ALLOANTIGENS

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The study of the active mechanisms of creation and maintenance of tolerance has led to the discovery of suppressor cells in some forms of transplantation tolerance [9, 10, 13]. However, the role of suppressors in tolerance induced with cyclophosphamide (CP) has so far received only little study. On the one hand, antigen-specific suppressor T cells have been demonstrated in tolerance induced to sheep red blood cells (SRBC) with the aid of CP [2, 14], and activation of antigen-nonspecific suppressor cells has been found after administration of CP [11, 15]. On the other hand, many investigations have revealed high sensitivity of precursors of suppressor T cells and their inducers to CP [8].

The writers showed previously that after thymectomy and injection of a massive dose of allogeneic splenocytes and CP, lasting (at least 8 months) tolerance to an allogeneic heart graft arises in the majority of adult CBA mice [3, 5]. Lymphocytes of tolerant animals were specifically areactive to the donor's alloantigens in mixed lymphocyte culture (MLC) [5] and in the graft versus graft reaction (GVGR) [6].

The aim of this investigation was to study the possible role of suppressor mechanisms in the observed form of tolerance.

EXPERIMENTAL METHODS

Adult male CBA ($H\text{-}2^k$), C57Bl/6 ($H\text{-}2^b$), and BALB/c ($H\text{-}2^d$) mice and (CBA × C57Bl/6)F_1 hybrids, obtained from the Stolbovaya nursery, Academy of Medical Sciences of the USSR, were used in the experiments. The (CBA × BALB/c)F_1 hybrids were specially bred by the writers.

Adult CBA mice were thymectomized by the method described previously [3, 5] and, 3-4 weeks later, they were given an intravenous injection of a massive dose (10^8) of spleen cells of C57Bl/6 mice, followed (after 18-24 h) by a single intraperitoneal injection of 200 mg/kg CP. The reactivity of cells of the tolerant mice was studied 1-2 months after the induction of tolerance.

To carry out the GVGR, the ability of spleen cells of intact CBA mice to prevent the immune response to SRBC of spleen cells from (CBA × C57Bl/6)F_1 hybrid mice, presensitized to SRBC, during combined adoptive transfer into CBA mice irradiated in a dose of 9 Gy, was used. Simultaneously with transfer of the splenocytes, the recipients received an intravenous injection of $5 \cdot 10^8$ SRBC. The immune response of the (CBA × C57Bl/6)F_1 hybrids to SRBC was determined in the spleen of irradiated recipients 5 days after adoptive transfer, using the local hemolysis in gel method. For the GVGR either 10^7 splenocytes of intact CBA mice or 10^6 splenocytes of CBA mice sensitized 5 days before the experiments by intravenous injection of 10^8 spleen cells of C57Bl/6 mice, or 10^7 splenocytes of CBA donor mice tolerant

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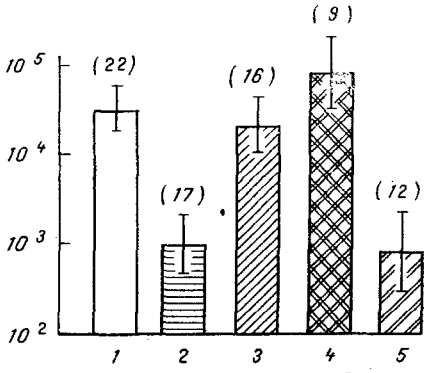

Fig. 1. Specific areactivity of spleen cells of mice tolerant to C57Bl/6 (H-2^b) alloantigens, detectable by GVGR. To set up the GVGR in CBA mice irradiated with a dose of 9 Gy, spleen cells of various donors were injected intravenously together with $5 \cdot 10^8$ SRBC: group 1) $3 \cdot 10^7$ splenocytes (CBA × C57Bl/6)F_1; group 2) 10^7 splenocytes of intact CBA mice + $3 \cdot 10^7$ splenocytes (CBA × C57Bl/6)F_1; group 3) $2 \cdot 10^7$ splenocytes of CBA mice tolerant to C57Bl/6 + $3 \cdot 10^7$ splenocytes (CBA × C57Bl/6)F_1; group 4) $3 \cdot 10^7$ splenocytes (CBA × BALB/c)F_1; group 5) $2 \cdot 10^7$ splenocytes (CBA × BALB/c)F_1. Here and in Fig. 2, mean values of number of AFC to SRBC in spleen of irradiated recipients 5 days after adoptive transfer of splenocytes of different donors are given; number of mice shown in parentheses.

to C57Bl/6 antigens were injected. Either (CBA × C57Bl/6)F_1 splenocytes or (CBA × BALB/c)F_1 splenocytes ($3 \cdot 10^7$ cells), injected together with CBA cells, served as the target for GVGR. The F_1 donors were immunized 7-30 days before the experiment with 10^6 SRBC (intravenously).

Suppressor activity of the spleen cells of mice tolerant to antigens of C57Bl/6 mice was determined by their ability to inhibit the GVGR, involving splenocytes of intact CBA mice. During adoptive transfer in this case, a triple mixture was injected into the irradiated CBA recipients: $3 \cdot 10^7$ spleen cells of (CBA × C57Bl/6)F_1 hybrids, primed for SRBC, 10^7 spleen cells of intact CBA mice, and $2 \cdot 10^7$ splenocytes of mice tolerant to C47Bl/6 antigens. To determine that the suppressors belonged to the T cell population, spleen cells of tolerant mice were treated with anti-T-globulin (ATG) and complement in vitro [12] before addition to the cell mixture to be injected into irradiated recipients.

EXPERIMENTAL RESULTS

Using splenocytes of CBA (H-2^k) mice, tolerant to C57Bl/6 (H-2^b) antigens instead of intact CBA mice for the GVGR, we determined their inability to inhibit the response of adoptively transferred cells of (CBA × C57Bl/6)F_1 hybrids to SRBC. The results given in Fig. 1 show that spleen cells of (CBA × C57Bl/6)F_1 hybrid mice primed for SRBC produced antibodies to SRBC intensively in irradiated CBA recipients (group 1 – positive control). A mixture of cells of these hybrids with splenocytes of intact CBA, injected into irradiated CBA recipients, gave a sharp decrease in the number of antibody-forming cells (AFC) in the recipients' spleen (groups 1 and 2). Cells of CBA mice receiving tolerogenic treatment in relation to H-2^b antigens did not induce a decrease in the number of AFC in the GVGR in the spleen of the irradiated recipients during their combined injection with cells of (CBA × C57Bl/6)F_1 hybrids (group 3). These results are evidence of the areactivity of the lymphocytes of tolerant mice toward target cells with antigens of the H-2^b allotype. This areactivity is specific, for splenocytes of tolerant mice themselves can inhibit the response of lymphocytes of (CBA × BALB/c)F_1 hybrids (groups 4 and 5).

These results are similar to those obtained by the writers previously when studying transplantation tolerance induced in CBA mice after receiving an injection of 200 mg/kg CP

TABLE 1. Removal of Suppressor Activity of Spleen Cells of CBA Mice Tolerant to Neonatal Transplantation of the Hearts of C57Bl/6 (H-2^b) Mice as a Result of Their Treatment with ATG and Complement

Group No.	Donors of cells			No. of mice	AFC to recipients' spleens
	CBA, tolerant to C57Bl/6	CBA, presensitized to C57Bl/6	(CBA×C57Bl/6) primed for SRBC		
	number of cells				
	10^7	$2 \cdot 10^8$	$3 \cdot 10^7$		
1	—	—	+	8	61,455 (42 945—88 123)
2	—	+	+	8	2,028 (653—6294)
3		Treated with ATG and complement			
	—	+	+	7	50,193 (33 007—76 454)
4	—	—	+	7	79,193 (65 804±95 445)
5	Treated with normal globulin and complement +	+	+	6	17,544 (13 597—22 623)
6	Treated with ATG and complement +	+	+	6	7,521 (6 559—8 612)

Legend. Donors' splenocytes injected intravenously together with $5 \cdot 10^8$ SRBC.

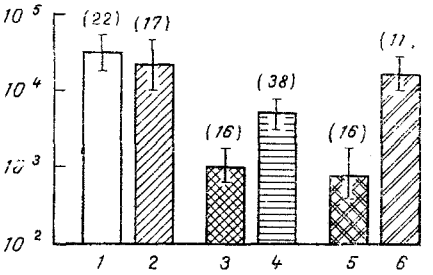

Fig. 2. Suppressor activity of spleen cells of mice tolerant to C57Bl/6 (H-2^b) alloantigens, detected by GVGR. Splenocytes of various donors injected intravenously together with $5 \cdot 10^8$ SRBC into irradiated (9 Gy) CBA recipients: group 1) $3 \cdot 10^7$ splenocytes (CBA × C57Bl/6)F_1; group 2) $2 \cdot 10^7$ CBA splenocytes tolerant to C57Bl/6 + $3 \cdot 10^7$ splenocytes (CBA × C57Bl/6)F_1; group 3) 10^7 splenocytes of intact CBA mice + $3 \cdot 10^7$ splenocytes (CBA × C57Bl/6)F_1; group 4) $2 \cdot 10^7$ splenocytes of CBA mice tolerant to C57Bl/6 + 10^7 splenocytes of intact CBA mice + $3 \cdot 10^7$ splenocytes (CBA × C57Bl/6)F_1; group 5) 10^8 splenocytes of CBA mice presensitized to C57Bl/6 + $3 \cdot 10^7$ splenocytes (CBA × C57Bl/6)F_1; group 6) $22 \cdot 10^7$ splenocytes of CBA mice presensitized to C57Bl/6 + $3 \cdot 10^7$ splenocytes (CBA × C57Bl/6)F_1 (suppression of GVGR).

3 h before transfer of 10^8 splenocytes of (CBA × C57Bl/6)F_1 hybrids [1]. Specific areactivity of the splenocytes relative to the donor's antigens was found in the mouse chimeras in the local graft versus host reaction and their lymphoid cells did not respond by blast transformation to cells of (CBA × C57Bl/6)F_1 hybrids in MLC [1, 2]. Splenocytes of the chimeras significantly inhibited the response of lymphocytes of CBA mice to (CBA × C57Bl/6)F_1 stimulating cells in MLC [1, 2].

Comparison of the two forms of tolerance to the allograft – semiallogeneic cyclophosphamide chimeras and the model of tolerance to alloantigens elaborated by ourselves and obtained in adult animals in response to the combined action of thymectomy, CP, and a massive dose of allogeneic splenocytes – in both cases revealed specific areactivity in relation to cells of the donor's line. This areactivity of peripheral lymphocytes is characteristic of models of tolerance in which long-term survival of allografts was observed [7, 10].

Considering the important role of active mechanisms in the maintenance of tolerance to a graft [7, 9, 10, 13] and the presence of suppressor cells in semiallogeneic chimeras [1, 2], we attempted to use the GVGR to detect and characterize suppressor cells in the spleen of CBA mice with induced tolerance to C57Bl/6 antigens, unaccompanied by chimerism of lymphoid tissue.

Suppressor activity of the spleen cells of the tolerant animals was assessed on the basis of their ability to inhibit the GVGR, induced by intact CBA splenocytes (Fig. 2, groups 3 and 4). These cells were able to inhibit rejection caused not only by intact CBA, but also presensitized "CBA-anti-C57Bl/6" splenocytes (Fig. 2, groups 5 and 6). A mixture of splenocytes of tolerant mice with intact CBA lymphocytes in the ratio of 2:1 led to a fivefold increase in the number of AFC compared with the group in which a GVGR took place (Fig. 2, groups 3 and 4). However, the number of transferred suppressors was insufficient to prevent killing totally (Fig. 2, groups 1 and 4). Presensitized CBA lymphocytes were more effective in suppressing (CBA × C57Bl/6)F_1 target cells (Fig. 2, group 5). This enabled the ratio of suppressor to killer cells to be increased to 10:1 (Fig. 2, group 6) and 5:1 (Table 1). The results show that in ratios of 5:1 and 10:1 the suppressors increased the number of AFC produced by splenocytes of (CBA × C57Bl/6)F_1 hybrids by an order of magnitude (Fig. 2, groups 5 and 6).

Treatment of spleen cells of tolerant mice with ATG and complement before adoptive transfer effectively weakened suppression (Table 1, groups 5 and 6). However, the residual amount of AFC was still much greater than in recipients receiving only a mixture of "killer" cells and (CBA × C57Bl/6)F_1 targets (Table 1, groups 6 and 2). The fact that suppression was not completely abolished after treatment of the suppressor cells with ATG and complement was not due to low ATG activity, for treatment of presensitized CBA cells with this ATG under the conditions used to abolish suppressor activity, completely abolished activity of the killer T cells (Table 1, group 3).

It can thus be concluded from these results that specific areactivity of the tolerant animals was largely due to the presence of suppressor cells carrying the marker of T lymphocytes. The method of induction of tolerance which we used included as an essential step thymectomy of adult animals. The suppressor cells which we discovered 2-4 months after the operation therefore must belong to the population of long-living T lymphocytes. The cotolerogenic effect of thymectomy may probably be attributed to two factors: first, a change in the ratio between short- and long-living subpopulations of T cells [4] and, second, the fact that thymectomy prevents release from the state of areactivity, for it prevents maturation of immunocompetent cells from immature precursors.

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HUMORAL MECHANISM OF INHIBITION OF IMMUNOREACTIVITY OF MOUSE LYMPHOCYTES IN VITRO BY MASTOCYTOMA P815 CELLS

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Immunosuppression in the wide sense of the term is one of the most important problems in antitumor immunity. The "escape" of tumor cells (TC) from "surveillance" of the immune system can be attributed to at least two causes. First, TC may inhibit the activity of effector antitumor lymphocytes. We know that lymphocytes which infiltrate a tumor are characterized by sharply reduced functional activity [7, 8]. Second, during growth of a syngeneic tumor in vivo suppressor cells which prevent generation of cytotoxic T lymphocytes [1, 6, 10, 12] and inhibit their function [3, 9] are activated. Evidence has been obtained that TC produce immunosuppressor factors (ISF) which directly inhibit the activity of effector lymphocytes [2, 5, 11, 13] and (or) activate suppressor T cells [4].

This paper describes an attempt to assess the ability of mastocytoma P815 cells and a supernatant of their culture fluid (SN) to inhibit proliferation of the splenocytes of DBA/2 mice in the blast transformation reaction (BTR) and in mixed lymphocyte culture (MLC) and to study the effect of preliminary treatment of splenocytes with SN on their functional activity.

EXPERIMENTAL METHODS

Male DBA/2 (H-2^d) and C57Bl/6 (B6, H-2^b) mice aged 2-4 months were used. The mastoma P815 was maintained in vitro. To obtain the SN, P815 cells were cultured for 72 h, after which the SN was collected by centrifugation and kept at −20°C. The following culture medium (CM) was used for the functional tests: RPMI 1640 (Flow Laboratories, England), with the addition of L-glutamine (2 mM), HEPES-buffer (25 mM, Flow Laboratories), inactivated human group IV serum (2.5%), 2-mercaptoethanol ($5 \cdot 10^{-5}$ M; Serva, West Germany), and gentamicin (50 μg/ml). The BTR was carried out by culturing DBA/2 spleen cells ($250 \cdot 10^3$ per well) with concanavalin A (5 μg/ml, Flow Laboratories) in round-bottomed 96-well micropanels (Linbro, England) for 72 h at 37°C in an atmosphere with 5% CO_2. To set up the MLC the cells were cultured in flat-bottomed 96-well micropanels (Flow Laboratories) for 5 days at 37°C in an atmosphere with 5% CO_2, with a ratio of responding cells (DBA/2 splenocytes; $250 \cdot 10^3$ per well) to stimulators (B6 spleen cells treated with mitomycin C) of 1:3. As the third component in BTR and MLC, P815 cells treated with mitomycin C (DBA/2 splenocytes in the control) or different dilutions of SN were added. The cells were treated with mitomycin C in a dose of 50 μg/ml at 37°C for 40 min, and then washed 3 times. ^{3}H-Thymidine (specific activity 24 Ci/mmole, 1 μCi per well) was added to the BTR 6 h before the end of culture. To study cell proliferation in MLC, 1 μCi of ^{3}H-thymidine (specific activity 4 Ci/mmole) was added to each well 18 h before the end of culture. The samples were harvested on glass fiber filters (Flow Laboratories) by means of a harvester, and radioactivity was measured on a β-spectrometer. Preliminary treatment of the DBA/2 mouse spleen cells with SN was carried out for 2 h at 37°C (dilution of SN 1:1, pretreatment with CM in the control) was carried out for

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