A First or Dominant Immunization. II. Induced Immunoglobulin Carries Transforming Growth Factor β and Suppresses Cytolytic T Cell Responses to Unrelated Alloantigens

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Summary

Fresh sera from mice immunized by bearing an immunogenic tumor or by repeated injections of allogeneic spleen cells or xenogeneic erythrocytes powerfully suppress cytolytic T cell responses in one-way mixed lymphocyte cultures. Suppression is not antigen specific, though is mediated by immunoglobulin (Ig)G specific for the immunizing antigen. Suppression caused by IgG mimics that caused by active transforming growth factor β (TGF-β). IgG associates with or carries latent TGF-β; however, suppression caused by the complex of IgG–TGF-β requires macrophages (Mφ), whereas active TGF-β alone does not. Also, IgG dissociated from TGF-β does not cause suppression, suggesting that Mφ may take up Ig–TGF-β, process the complex, and deliver active TGF-β to lymphocytes. Indeed, suppression by immune serum was prevented by antibody to Fc receptors, by saturating Fc receptors with heterologous IgGs, and by antibodies against TGF-β. The overall findings reveal a previously unrecognized regulatory circuit whereby IgG produced in response to one antigen nonspecifically downregulates cytolytic T lymphocyte responses to unrelated antigens. The findings introduce the intriguing possibility that TGF-β delivered by IgG and processed by Mφ may mediate important biological effects in processes such as wound healing, tumor growth, and some autoimmune diseases.

A prior or dominant immunization of mice with one antigen abolished $CD8^+$ cytolytic T lymphocyte (CTL)[1] responses to a second unrelated antigen given simultaneously (1). Suppression became systemic and could be transferred passively to normal mice by fresh immune serum alone. In the present experiments, fresh serum from such immunized mice in high dilution abolished CTL responses in one-way MLC, presumably in a way analogous to that occurring in vivo. The following experiments were designed to determine the nature of the serum components and the cells responsible for suppression of CTL in MLC.

Materials and Methods

Mice and Cell Lines. Donors of sera and lymphoid cells were C3H ($H2^k$) female mouse mammary tumor virus–negative (MTV^-) pathogen-free mice 8–12 wk old and housed in a barrier facility; donors of allogeneic cells were BALB/C ($H2^b$) female mice also pathogen free and housed in a barrier facility. C3H SCID mice purchased from Imdyne (San Diego, CA) were *Pneumocystis carinii* free and housed similarly. Tumor targets for measuring CTL in MLC were P815 (H-2^d) cells (see accompanying manuscript [1] for details).

Immunizations and Murine Sera. C3H mice under light ether anesthesia were injected in hind foot pads with 0.05 ml of 5% (vol cells/vol diluent) cell suspension of washed sheep or horse erythrocytes (SRBC or HRBC); injections were repeated every other day four to six times; mice were bled 1 or 2 d after the last injection; i.e., 7–12 d after injections began. Sera from immune mice (αSer) were pooled from three or four identically injected mice and used within 72 h unless noted otherwise. Normal mouse sera (NMS) were obtained from nonimmunized mice of the same age and housed identically. NMS and αSer were either untreated (Table 1, Exps. 1 and 2) or diluted 1:10 in RPMI and separated into <100- and >100-kD fractions by filtration/centrifugation using Centricon Microconcentrators (Amicon, Beverly, MA) for Exp. 3 (Table 1) and all subsequent experiments. Both the <100- and >100-kD fractions were reconstituted to the same volume as the sample before fractionation and were considered to be at a dilution of 1:10.

Lymphocytes, Nonadherent Cells (Ad^-), Dendritic Cells, and Cells without Macrophages. Popliteal lymph node or spleen cells were dispersed and washed in complete medium; debris and aggregates were removed by gravity sedimentation. Ad^- were incubated twice for 60 min with carbonyl iron (10^8 cells/10 ml medium/2 gm carbonyl iron); ∼95% of macrophages (Mφ) and dendritic cells (DC) are removed by this procedure. Cells highly enriched for DC

[1] *Abbreviations used in this paper:* AD^-, nonadherent cells; αSer, sera from immune mice; CTL, cytolytic T lymphocytes; DC, dendritic cells; HRBC, horse red blood cells; Mφ, macrophages; NMS, normal mouse serum.

were obtained from cells that adhere to plastic culture plates (10^8 cells/10 ml medium/10-cm culture plates) in an initial 2-h incubation; nonadherent cells were removed and the volume restored. Most DC detach during an additional 18–22-h incubation while most Mϕ remain adherent; $\sim 2 \times 10^5$ detached cells are usually recovered per 10^8 spleen cells and 60–80% of these cells are DC with most of the contaminating cells consisting of Mϕ. Ad^- cells do not respond in MLC unless restored by adding DC, $\sim 3 \times 10^4$ DC/5×10^5 responding lymphocytes being optimal.

MLC and Assay for Cytolytic T Cells. MLC were 5×10^5 C3H ($H2^k$) normal spleen cells and 5×10^5 normal, irradiated (2,000 rad) BALB/c ($H2^d$) spleen cells per well of 96-well flat-bottomed tissue culture plates (2). Cells were suspended in 100 μl of complete medium, which was: RPMI 1640 with 25 mM Hepes supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 5×10^{-5} M 2-ME, and 10% FCS; 50 U penicillin and 50 μg of streptomycin were added per 100 ml of medium. 100 μl of supplemented RPMI 1640 without FCS was added to each culture; this addition was either medium alone for control cultures or contained agents such as IgGs, TGF-β, etc. Thus, the final concentration of FCS was 5%, and concentrations or dilutions of each additional agent are recorded per milliliter of the final 200 μl of culture medium. Cultures were incubated 4 or 5 d at 37°C, 5% CO_2, and 100% humidity. Controls were six or eight replicate cultures to which medium alone was added; each variable was tested in duplicate or triplicate cultures. Viable cell recovery was equivalent (less than twofold difference) in control and variously treated cultures. One-half of each culture (E/T cell ratio of ~50:1) and seven additional double dilutions of each culture were assayed separately against ^{51}Cr-labeled P815 ($H2^d$) target cells using 4-h ^{51}Cr release assay (2); each culture was assayed separately so that results are means for six to eight cultures containing medium only and two or three cultures for each variable tested. For convenience for comparing control and variously treated cultures, results are reported for only three fourfold dilutions or fractions of cultures assayed. Because the slopes of curves for lysis caused by different dilutions of cells from test and control cultures are rarely parallel, we have no adequate way for statistical analysis of differences. Also, for this reason, it is not strictly appropriate to convert results to lytic units, although we have done this to make comparing gross differences easier. For this purpose, a lytic unit is arbitrarily defined as the reciprocal of the fraction of identical cultures, rounded to the nearest 10, causing 30% lysis of labeled target cells. We only emphasize differences between test and control cultures greater than fourfold whether indicated by comparing lysis at different fractions of culture or by lytic units. Furthermore, all of the differences of this magnitude reported have been confirmed in two or more additional experiments, which are not reported. Treating responder cells before culture or treating cells harvested from MLC after 4 or 5 d incubation with anti-CD8 antibody and complement abolishes CTL activated in this culture system (2).

Secretion and Assay for TGF-β. Lymphoid cells or a murine cell line designated 7.63, originally designated 1591-N-TGF-1, transfected to secrete murine latent TGF-β1 (3) were cultured in 5×10^5 cells/ml of MEM containing 0.1% FCS at 37°C, 7.5% CO_2, and 100% humidity for 48 h. The medium alone contained <0.01 ng active and <0.1 ng total TGF-β/ml. Supernatants were assayed without treatment to measure active TGF-β, and after acidification at pH 2.0 for 30 min, to measure total TGF-β. Secretion is recorded as ng/10^6 cells per 24 h using an assay dependent on inhibition of proliferation of cells from a line of mink lung "epithelial-like" cells, designated MvlLu (4). TGF-β is secreted as a latent complex of ~110 kD; only the cleaved active 25-kD homodimer binds receptors or is bound by the anti-TGF-β antibodies we have. The assay does not discriminate between different isoforms of TGF-β, but neutralization of suppression by antibodies to TGF-β confirms that suppression is mediated by TGF-β, and antibodies relatively specific for different isoforms can indicate the form of active TGF-β in a preparation using the Mv1Lu assay.

Antibody Titers. Sera were serially double diluted in PBS containing 0.5% BSA in 96-well V-bottomed plates. For agglutinin titers, each well contained 50 μl of diluted serum and 50 μl of 0.05% RBC; for hemolysin titers, each well contained an additional 50 μl guinea pig complement absorbed with SRBC and HRBC and diluted 1:20 in PBS; each sample was titered in duplicate for both agglutinin and hemolysin titers. Plates were incubated 1 h at 37°C and overnight at 5°C. Titers were read using a magnification of 3. The antibody titers are reported as the reciprocal of the highest serum dilution that caused gross agglutination or complete hemolysis of erythrocytes; duplicate titers did not vary more than one double dilution. Hemolysin titers are not reported separately but were equal to or one double dilution lower than agglutinin titers.

Reagents. Murine TGF-β1 was the supranatant of a murine tumor cell line transfected with cDNA for murine TGF-β1, designated 7.63; the supranatant contains ~10 ng/ml of TGF-β after acid activation and virtually no active TGF-β without acid treatment. Because both latent and active TGF-β adhere to plastic, all samples are processed and stored in plasticware preincubated with 1% BSA in saline. The total amount of TGF-β in such samples is stable during storage for many months. The following reagents were purchased: porcine TGF-β1 (pTGFβ) (lot no. BO60; R & D Systems, Minneapolis, MN); monoclonal mouse anti-TGF-β2, β3 (lot no. B1674; Genzyme, Cambridge, MA); TGF-β neutralization antibody, primarily for TGFβ1 (Chicken) (lot no. W247; R & D Systems); normal chicken IgY (endotoxin free) (lot no. U142; R & D Systems). Anti-Fc receptor antibody (rat anti–mouse monoclonal FcR, designated 2.4G2) was a gift of Dr. J. Quintans (University of Chicago); the supranatant and rat control Ig were preincubated with responding lymphocytes for 30 min before stimulator cells and immune sera were added to culture. Murine (m)IgG chromatographically purified were purchased from Cappel, Organon Teknika Corp. (Durham, NC); and from Zymed Laboratories, Inc. (San Francisco, CA). Preparations of mIgs contained 0.05% Na azide, which was removed by repeated high dilution and reconcentrating using Centricon 3 microconcentrators. Affinity-purified goat antibody to mouse Ig (IgG, IgA, IgM) was purchased from Cappel, Organon Teknika Corp. Rabbit IgG was affinity-isolated antigen-specific antibody against horse spleen ferritin (Sigma Chemical Co., St. Louis, MO). Sterile sheep and horse blood in Alsever's solution was purchased from Environmental Diagnostics (Burlington, NC), and was used within 6 wk.

Absorptions of Sera. Sera were absorbed on Protein A–Sepharose® 4 Fast Flow (Pharmacia Fine Chemicals, Piscataway, NJ); on protein G using MabTrap™ G and HiTrap™ G (Pharmacia LKB Biotechnology, Uppsala, Sweden), and on affinity-purified goat antibody to murine Ig (IgG, IgA, IgM) coupled to CN-Br Sepharose 4B (Pharmacia Fine Chemicals), 1 mg antibody/ml of Sepharose beads. Sera, diluted 1:10 in 0.2 M Na_2PO_4 and beads (vol/vol), were constantly mixed for 75 min at 20°C. The supranatants obtained after centrifugation and filtration were dialyzed against medium overnight. For absorption on heterologous erythrocytes, sera were diluted 1:25 in medium and absorbed (vol/vol) with packed erythrocytes for 10 min at 5°C with frequent mixing. The supranatant was obtained by centrifugation and the procedure was repeated.

Results

Immune Sera Abolish CTL Responses in MLC. We observed repeatedly that fresh NMS added to MLC at final dilution in culture of 1:50–100 had no significant effect on CTL responses; in contrast, fresh immune sera (αSer) at these or higher dilutions abolished CTL responses. In different experiments αSer were from: (*a*) mice bearing immunogenic tumors that had grown for 2 or 3 wk or were from mice that had recently rejected such tumors; or (*b*) mice bled 1–3 d after receiving three or more injections of allogeneic spleen cells or xenogeneic erythrocytes given every 2 or 3 d. These observations were confirmed in a single experiment (Table 1, Exp. 1). We found repeatedly, as in Table 1, Exp. 1, that αSer raised against xenogeneic erythrocytes were usually suppressive at higher dilutions than were sera from mice immunized by tumors or allogeneic spleen cells. Partially for this reason, but also for convenience for presenting data, we report in the following experiments only results using sera from C3H mice immunized with xenogeneic erythrocytes, though the essential findings have been confirmed using sera from C3H mice immunized in these other ways and sera from two other strains of mice immunized with xenogeneic erythrocytes.

Blood was as suppressive as serum (Table 1, Exp. 2), indicating that the process of clotting does not activate or generate factors responsible for suppression. For this experiment, 0.1 ml of heart blood from a single normal or immune (five injections of SRBC) mouse was added directly to 4.9 ml of culture medium and cells were immediately removed by centrifugation. Serum was prepared from the remaining heart blood obtained from the same mice after allowing clot retraction to occur at room temperature for 90 min. The experiment included blood and sera from a mouse injected identi-

Table 1. *Suppression of CTL Responses in MLC by Immune Sera*

			Cytotoxicity			
			Percent ^{51}Cr release			
Exp.	Addition*	Dilution	1/8‡	1/32	1/128	Lytic units
1	Medium	–	88	75	44	256
	NMS	1:100	81	70	34	256
	TBA Ser.	1:100	34	16	4	8
	αC57 Ser.	1:100	23	10	3	8
	αSheep Ser.	1:300	27	11	2	8
2	Medium	–	100	90	59	512
	Normal blood	1:900	97	68	44	512
	αSheep blood	1:900	18	19	10	8
	αSheep Ser.	1:900	5	5	12	<1
3	Medium	–	80	59	23	128
	NMS	1:600	86	26	40	128
	αSheep Ser.	1:600	1	0	2	<1
	SCID Ser.	1:200	77	76	28	128
	SCID "αSheep Ser."	1:200	86	83	43	128
4	Medium	–	89	78	48	256
	αSheep Ser.	1:300	1	0	0	<1
	αSheep >100 kD	1:300	1	0	0	<1
	αSheep <100 kD	1:300	96	92	68	>512

* In Exp. 1, TBA ser. was a pool from three C3H mice bearing immunogenic tumors, designated PRO4L, that had grown progressively for 3 wk and were ~1.5 cm in diameter. αC57 ser. was a pool from three C3H mice each injected in foot pads with 10^7 C57 spleen cells three times every 2 d and bled 1 d after the final injection. αSheep Ser. was from mice injected identically with 10^8 SRBC and bled at the same time as mice receiving allogeneic cells. See text for Exps. 3 and 4. Sera were unfractionated in Exps. 1, 2, and 3.
‡Fraction of culture.

cally with HRBC; results were virtually the same as those for immune anti–sheep blood and serum (data not shown). Fresh serum from SCID mice, whether untreated or injected repeatedly with SRBC as in Exp. 2, caused no suppression (Table 1, Exp. 3), suggesting that suppression was most likely due to a product of an immune response.

In a single trial all suppressive activity of an αSer fractionated by gel filtration was recovered in fractions of >70 kD. In repeated trials all suppressive activity of different pools of αSer was recovered in the >100-kD fraction obtained by filtration/centrifugation (Table 1, Exp. 4), and as in that experiment the <100-kD fraction usually stimulated responses two- to fourfold. To reduce complications caused by components in sera that may have contrary effects in MLC, all sera for the following experiments were separated by filtration/centrifugation and only the >100-kD fractions were used, except as noted.

We observed repeatedly that sera from mice injected with antigen five or six times were more suppressive than sera from mice injected three or four times, and that suppressive activity of sera was often lost or decreased by more than a threefold dilution when stored for 1 wk or more at 5 or −20°C, though we have not assessed these variables systematically. To avoid such problems αSer were used within 48 h of bleeding in each of the following experiments, and each serum tested was the >100-kD fraction of serum pooled from three or more mice injected five or six times with xenogeneic erythrocytes and bled 1 or 2 d after the last injection. We have found subsequently that the >100-kD fraction of αSer stored at −80°C retains full suppressive activity for at least 6 wk. In each of the following experiments, αSer and each of the variables were tested at two or usually three threefold serial dilutions; we report results for only the highest serum dilution, which caused ⩾10-fold suppression of CTL responses.

IgG Specific for the Immunizing Antigen Causes Suppression. As shown in Table 2, Exps. 1 and 2, αSer absorbed on protein A, protein G, or goat anti–murine Ig coupled to Sepharose no longer caused suppression. Protein A and protein G bind predominantly IgG whereas the goat anti–murine Ig should remove all isotypes. Indeed, considerable anti-SRBC antibody (IgM and IgA identified by ELISA; data not presented) remained after absorption on protein A or protein G, but not after absorption on the goat anti–murine Ig. In recently completed experiments, absorption of αSer on goat anti–murine IgG coupled to Sepharose was as effective in removing all suppressive activity as absorption on goat anti–murine Ig (IgM, G, A), though absorption on αIgG did not remove all specific anti-RBC antibody, whereas absorption on αIg did. Absorption of αSer on goat anti–murine IgM coupled to Sepharose reduced antibody titers four-

Table 2. *Absorption and Elution of Ig Causing Suppression*

Exp.	Additions to cultures*	Absorption on:	Cytotoxicity: Percent ^{51}Cr release 1/8‡	1/32	1/128	Lytic units	Antibody titer: αSheep	αHorse
1	Medium	–	82	72	32	128	–	–
	αSheep Ser.	–	2	3	0	4	960	–
	αSheep Ser.	Protein A	81	74	34	128	240	–
	αSheep Ser.	αmIg	82	71	39	128	0	–
2	Medium	–	62	25	7	32	–	–
	αSheep Ser.	–	14	2	1	<1	1,280	–
	αSheep Ser.	Protein G	71	30	9	32	400	–
3	Medium	–	84	65	23	128	–	–
	αSheep Ser.	–	0	1	1	<1	1,600	0
	αSheep Ser.	SRBC	76	53	23	128	0	0
	αSheep Ser.	HRBC	2	3	4	<1	800	0
4	Medium	–	100	87	62	256	–	–
	αHorse Ser.	–	8	12	6	<1	0	3,200
	αHorse Ser.	SRBC	27	16	0	8	0	3,200
	αHorse Ser.	HRBC	87	50	30	128	0	0

* All sera were >100-kD fractions tested at dilutions of 1:300 (Exps. 1 and 2) and 1:200 (Exps. 3 and 4); absorbed samples were tested at the same dilutions as the unabsorbed samples.
‡Fraction of culture.

to eightfold, but did not reduce suppressive activity of sera. Absorptions of immune or normal sera on goat IgG coupled to Sepharose or on Sepharose alone did not alter antibody titers, increase CTL responses, or alter the capacity of αSer to suppress, indicating that absorptions did not introduce extraneous factors that might affect cultures. It was unlikely that suppression was due to IgE since protein A removed suppressive activity, and heat inactivation of αSer at 56°C for 30 min did not reduce suppressive activity (data not presented). Together, the results indicated that suppression was mediated by IgG.

Table 2, Exps. 3 and 4, show that Ig directed against the antigen used to raise the immune serum was responsible for suppression; i.e., αSer raised against SRBC lost all specific antibody activity and all suppressive activity after absorption on SRBC but not after absorption on HRBC; similarly, αSer raised against HRBC lost all specific antibody activity and nearly all suppressive activity after absorption on HRBC but not after absorption on SRBC.

Antibodies against TGF-β Prevent Suppression. Nonantigen-specific, non-H2-restricted suppression caused by αSer mimicked suppression caused by active TGF-β in several ways. Addition of 1.0–10.0 ng active pTGF-β1 or mTGF-β1 to MLC powerfully suppressed CTL responses. Addition of this amount or 10× more latent mTGF-β had no effect on CTL responses. Maximum suppression occurred when αSer or active TGF-β was added to MLC during the first 24 h of culture; no suppression occurred when either agent was added on days 3 or 4 of culture. Also, both agents caused little or no suppression of CTL responses using sensitized cells obtained from mice recently immunized with the same alloantigen as used in the MLC.

To test directly whether TGF-β might be involved, antibodies to TGF-β were added to MLC. In repeated experiments a murine mAb against TGF-β 2,3 (IgG2b) at concentrations that partially neutralized the effects of 1.0–3.0 ng of active porcine or murine TGF-β prevented suppression caused by adding αSer to MLC (e.g., Table 3, Exp. 1). Control IgG2b of unknown specificity added to cultures at the same or 3× the concentration of the anti-TGF-β antibody had no effect on CTL responses. Also, in repeated experiments, a chicken antibody against TGF-β1 at concentrations that neutralized 1.0–3.0 ng of porcine or murine TGF-β in MLC very effectively prevented suppression caused by αSer, (e.g., Table 3, Exp. 2). The chicken antibody against TGF-β alone had no effect on CTL responses in MLC; however, the findings using chicken antibody have to be taken with reservation (and serve as a cautionary note) because the normal chicken Ig provided as a control caused bizarre effects on cells in MLC that we have not observed for the chicken antibody against TGF-β or for any other additive we have ever used during the course of a great many experiments.

Secretion of TGF-β by Lymphocytes. αSer might suppress CTL responses by stimulating cells in MLC to secrete TGF-β. For these experiments, secretion of TGF-β by lymphoid cells was compared with secretion by a murine cell line transfected

Table 3. *Antibodies against TGF-β Prevent Suppression*

Exp.	Additions to cultures*	αTGF-β Antibody‡	Cytotoxicity: Percent ^{51}Cr Release 1/8§	1/32	1/128	Lytic units
1	Medium	0	80	62	25	128
	Medium	αTGF-β2,3	82	66	31	128
	TGF-β1*	0	19	8	8	<8
	TGF-β1*	αTGF-β2,3	40	18	12	16
	αSer	0	6	2	2	0
	αSer	αTGF-β2,3	70	42	14	64
2	Medium	0	86	78	46	512
	Medium	αTGF-β1	81	74	40	512
	TGF-β1	0	19	6	2	8
	TGF-β1	αTGF-β1	77	40	14	128
	αSer	0	54	18	9	32
	αSer	αTGF-β1	84	82	40	512

* TGF-β was active pTGF-β1, 1.0 ng/ml (Exp. 1) and 3.0 ng/ml (Exp. 2). αSer were at dilutions of 1:200 (Exp. 1) and 1:300 (Exp. 2).
‡ Monoclonal murine anti-TGF-β2,3 (IgG2b), 50 μg/ml (Exp. 1). Murine IgG2b of unknown specificity used as a control at 50–200 μg/ml had no effect on responses in this or other experiments. Chicken anti-TGF-β1, 30 μg/ml (Exp. 2); see text for discussion of chicken Ig control.
§ Fraction of culture.

with cDNA for murine TGF-β1, which secretes ~10.0 ng TGF-β/10^6 cells per 24 h (3). Under the same culture conditions unfractionated lymphoid cells or highly purified populations of B or CD4+ cells secreted <1.0 ng TGF-β, but the addition of 1% murine serum caused unfractionated B or CD4+ cells to secrete 1.0–5.0 ng TGF-β/10^6 cells per 24 h. Stimulation of secretion of TGF-β was caused exclusively by the >100-kD fraction of sera. Secretion by B and CD4+ cells was always higher than for CD8+ cells. We found no complementation between populations that resulted in secretion of more TGF-β than could be accounted for by the separate populations. Adherent cells, predominantly Mφ but including DC, alone with or without added serum, did not secrete measurable amounts of TGF-β. We consistently found that supernatants of cells depleted of adherent cells and stimulated with serum contained more TGF-β than supernatants of cultures that included adherent cells, suggesting that adherent cells inhibited secretion or consumed TGF-β. Greater than 90% of TGF-β secreted by lymphocytes in cultures was latent and required acid activation for bioassay or causing suppression of CTL responses in MLC. Also, ~90% of the activity of the activated TGF-β was inhibited in the bioassay by chicken antibody–specific TGFβ1 (data not presented).

The essential findings found in many repeated experiments are shown in Table 4. Lymphocytes, including B cells, secrete significant amounts of latent TGF-β, and cells from normal mice secrete as much TGF-β as cells from immune mice. Furthermore, normal sera were as effective as αSer in stimulating secretion of TGF-β. In other experiments (data not presented),

Table 4. *Secretion of TGF-β by Lymphocytes*

Exp.	Lymphocytes		Serum*	TGF-β‡
1	B	Imm. LN	–	0.6
	B	Normal spleen	–	1.0
		–	αSer	0.6
		–	NMS	1.0
	B	Imm. LN	αSer	3.5
	B	Imm. LN	NMS	3.4
	B	Normal spleen	αSer	3.0
	B	Normal spleen	NMS	5.5
2	B	Imm. LN	–	0.7
	CD4	Imm. LN	–	0.3
	CD8	Imm. LN	–	0.5
			αSer	1.0
	B	Imm. LN	αSer	5.0
	CD4	Imm. LN	αSer	2.8
	CD8	Imm. LN	αSer	1.1

* All sera were at a dilution of 1:100 and were unfractionated.
‡ Total nanograms secreted/10^6 cells per 24 h; all preparations tested before acidification contained <0.1 ng active TGF-β.

sera from SCID mice or αSer absorbed on protein G were also as effective as normal or immune sera. Thus, it is unlikely that suppression of CTL caused by adding αSer to MLC is secondary to stimulation of secretion of TGF-β by responding cells. For this reason, we examined whether IgG in αSer might carry TGF-β.

TGF-β Is Associated with or Carried by IgG. Different individual or pooled NMS or αSer contained ~200–300 ng TGF-β/ml; >90% of serum TGF-β was latent and >90% of acid activated TGF-β in sera was neutralized by antibody to TGF-β1. We found no consistent differences in total amounts of latent or active TGF-β in either whole or the >100-kD fractions of NMS or αSer. Absorptions to remove Ig from NMS or αSer consistently reduced serum concentrations of TGF-β by ~25–50%. The findings indicated that ~2.0–10.0 ng of TGF-β is carried per 1.0 mg IgG (assuming that serum contains ~20 mg IgG/ml of serum), and each of two commercial preparations of mIgG affinity purified from "normal" mouse sera did contain 3–5 ng latent TGF-β/mg IgG (Table 5). Neither of the commercial preparations at a concentration of 1.0 mg IgG/ml culture medium inhibited (or enhanced) CTL responses. We then purified IgG from our NMS and αSer by absorption on protein G coupled to Sepharose, washing the column contents with 10 vol of diluent, pH 7.0, and briefly eluating absorbed IgG at pH 2.7 for 1.5 min using a 1.0 M glycine-HCl buffer. The neutralized eluate from NMS contained no measurable active TGF-β and ~5.0 ng latent TGF-β/mg IgG, which caused no suppression when added to MLC at 1.0 mg IgG/ml of culture medium. In marked contrast, the eluate from αSer, containing ~1.0 ng active and ~30.0 ng latent TGF-β/mg IgG, abolished CTL responses at a dilution that added ~3.0 μg IgG and ~0.1 ng TGF-β/ml of culture medium (Table 5, Exp. 3).

IgG Dissociated from TGF-β No Longer Causes Suppression. While IgG in αSer carried significant amounts of latent TGF-β, suppression by αSer appeared to be an order of magnitude greater than could be accounted for if all the carried TGF-β was activated. Indeed, αSer acidified at pH 2.0 for 30 min no longer suppressed CTL responses at the same high serum dilution, though acid-treated αSer at low dilutions that added 1.0–3.0 ng of the activated TGF-β/ml of culture medium did suppress, and this suppression was prevented by preincubation of the treated sera with antibody to TGF-β (data not presented).

Acid treatment of αSer not only activated TGF-β but also dissociated it from IgG. This was shown by acidifying αSer and fractionating it before neutralization. This procedure yielded all specific αSRBC or αHRBC antibody in the >100-kD fraction and all TGF-β in the active form and in the <100-kD fraction. The acidification/fractionation procedure did not cause any loss of complement-dependent hemolytic activity, which requires the Fc portion of antibody molecules. Interestingly, fractionation of αSer after acidification and neutralization yielded the same amounts of active TGF-β, but virtually all of the TGF-β was now recovered in the >100-kD fractions, indicating that the activated TGF-β reaggregates

Table 5. *IgG Carries TGF-β*

Exp.	Preparation‡	Source	TGF-β/IgG* Active	TGF-β/IgG* Total	Suppression of CTL/mg IgG§
			ng/mg		
1	IgG (commercial)	NMS	0.5	5.0	None, 1.0
2	IgG (commercial)	NMS	0.5	3.5	None, 1.0
3	IgG Prot. G eluate	NMS	0	5.0	None, 1.0
	IgG Prot. G eluate	αSer	1.0	30.0	Complete, 0.01

* Preparations tested before acidification (active) and after acidification (total).
‡ Commercial murine IgG: Exp. 1 (Cappel Laboratories) and Exp. 2 (Zymed Labs., Inc.), see Materials and Methods.
§ Per milliliter of culture medium.

possibly with itself and/or other large molecules. These findings for one experiment are shown in Table 6. Aliquots of αSer were either acidified for 30 min and fractionated before neutralization or were treated with the same quantities of acid and base added simultaneously before fractionation. The >100-kD fractions of both samples had the same antibody titers to SRBC but the >100-kD fraction of the acid-treated aliquot no longer suppressed effectively whereas the control aliquot did, suggesting that the combination of IgG and TGF-β in fresh immune serum may have to be in a particular or unique configuration for suppression to occur.

Macrophages Are Obligatory for Suppression Caused by Immune Serum. Two cell types are obligatory for $CD8^+$ CTL responses in MLC, $CD8^+$ lymphocytes, and DC (5). Selective removal in vitro of B lymphocytes, or in vivo of $CD4^+$ lymphocytes or NK cells from populations of responder and irradiated stimulator cells, did not lower (and usually caused higher) CTL responses, and in each case αSer was fully suppressive (data not shown). In contrast, αSer caused no suppression in Ad^- cultures but reconstituted with DC (Ad^- + DC) (Table 7, Exp. 1), though small quantities of active pTGF-β abolished responses in such cultures (Table 7, Exp. 2). These findings were confirmed in similar experiments using different αSer and active mTGF-β as well as pTGF-β. IgG–TGF-β might stimulate Mφ to secrete factors, including leukotrienes, prostaglandins, nitric oxide, or cytokines, that could suppress lymphocyte function, but in an extensive series of experiments we were unable to prevent suppression caused by αSer with indomethacin, acetylsalicylic acid, *N*-monoethyl-L-arginine, TNF-α, IL-2, or selected other cytokines, each tested at six concentrations over a 2-log range (data not shown).

Interference with Mφ Function Prevents Suppression by Immune Sera. Presumably Mφ take up IgG–TGF-β via Fc receptors for IgG and, as shown in Table 8, Exp. 1, a rat antibody directed against murine Fc receptors partially prevented suppression by αSer. Also, rabbit IgG added to cultures prevented suppression by αSer in a dose-dependent manner (Table 8,

Table 6. *IgG Dissociated from TGF-β No Longer Suppresses*

Suppression by:*	Treatment‡	Cytotoxicity: Percent Cr release 1/8§	1/32	1/128	Lytic units	Antibody titer
Medium	–	93	98	71	512	–
αSer	Control	21	10	6	<8	2,560
αSer	Acidification Neutralization	85	78	49	256	5,120

* The dilution of αSer was 1:300.
‡ Acidification was at pH 2.0 for 30 min with fractionation done before neutralization; control αSer was treated with the same quantity of acid and base added simultaneously before fractionation. Results are for the >100 kD fraction.
§ Fraction of culture.

Table 7. *Macrophages Are Obligatory for Suppression Caused by Immune Serum but Not for Suppression Caused by Active TGF-β*

Exp.	Responder and irradiated stimulator cells*	Additional cells‡	TGF-β or immune serum§	Cytotoxicity: Percent ^{51}Cr release 1/8‖	1/32	1/128	Lytic units
1	Whole	0	0	65	37	14	64
	Whole	0	αSer	13	5	3	<8
	Whole	DC	0	71	36	16	64
	Whole	DC	αSer	7	5	3	<8
	Ad⁻	0	0	14	5	12	<8
	Ad⁻	0	αSer	5	2	5	<8
	Ad⁻	DC	0	87	62	34	128
	Ad⁻	DC	αSer	77	60	23	128
2	Whole	0	0	73	46	17	128
	Whole	0	TGF-β1	5	5	9	<8
	Ad⁻	0	0	13	6	4	<8
	Ad⁻	DC	0	73	37	15	128
	Ad⁻	DC	TGF-β1	4	3	5	<8

* 5×10^5 responder and 5×10^5 irradiated stimulator cells, either whole or depleted of adherent cells (Ad⁻).
‡ 3×10^4 C3H DC added per culture.
§ αSer (1:300) or pTGF-β1 (3 ng/ml of culture medium).
‖ Fraction of culture.

Table 8. *Interference with Fc Receptors Prevents Suppression by Immune Sera*

Exp.	Suppression by:*	Additions‡	Cytotoxicity: Percent ^{51}Cr release 1/8§	1/32	1/128	Lytic units
1	Medium	–	100	88	49	512
	αSer	–	20	6	0	<8
	–	αFc AB	96	77	33	256
	–	Ig control	100	78	38	256
	αSer	αFc AB	93	47	9	128
	αSer	Ig control	51	17	2	32
2	Medium		85	80	62	512
	αSer		16	2	0	<8
	–	Rabbit IgG (50 μg/ml)	87	75	65	512
	–	Rabbit IgG (150 μg/ml)	88	80	65	512
	αSer	Rabbit IgG (50 μg/ml)	65	34	13	32
	αSer	Rabbit IgG (150 μg/ml)	84	65	40	256

* Dilutions of αSer were 1:400 (Exp. 1) and 1:300 (Exp. 2).
‡ In Exp. 1, the concentration of the αFcAB was ~15 μg/ml, and the rat Ig control ~10× the concentration of the αFcAB.
§ Fraction of culture.

Exp. 2), presumably by saturating Fc receptors. In addition, various "particulate" and soluble antigens that are phagocytized or taken up by other means also prevented suppression. For example, adherent cells were obtained by removing nonadherent cells after a 2-h incubation; the adherent cells were cultured alone or with a soluble antigen, Pc-KLH, 10 μg/ml of culture medium; the cells were incubated for 24 h and then washed thoroughly. These cells were compared with fresh adherent cells for restoring the capacity of αSer to suppress cultures lacking adherent cells but with added DC, as was done in Table 7, Exps. 1 and 2. The fresh adherent cells again completely restored the capacity of αSer to suppress; suppression was less using the adherent cells incubated for 24 h alone (possibly because of interaction of adherent cells with protein antigens in FCS?) and αSer caused no suppression using adherent cells incubated with the antigen (data not presented). Apparently, prior or other engagement of Mϕ as well as blocking Fc receptors may prevent these cells from taking up and/or processing IgG-TGF-β in αSer. Together, the findings suggest that suppression is not due to "activated Mϕ;" e.g., Mϕ stimulated with thioglycolate or lectins for ≥24 h that have many altered activities, including secretion of TGF-β (6).

Discussion

Fresh homologous sera are often "toxic" for many culture systems; the fact that our sera came from pathogen-free mice may account for why 1–2% NMS did not suppress cultures and allowed us to study components in immune serum that do suppress CTL responses. Though our immune sera were obtained from intensely immunized mice, immunization was meant to be comparable to the antigenic challenge that might occur with growing tumors or infectious agents. Adjuvants were not used and all of our sera were obtained within 6–12 d of the first injection of antigen; thus, we do not think our results are an artifact of an unphysiological regime for immunization. Though we have not studied the variables of antigen dose or injection schedule systematically, we have observed that serum obtained 7 d after a single injection of antigen was much less suppressive than sera from mice receiving the same total dose of antigen given every 2 d for three injections.

Repeated antigen injections should favor formation of circulating antigen–antibody complexes that are taken up by Mϕ and can cause various immunologic effects (7–9). Our αSer may well contain complexes present in great antibody excess and such complexes could play a role, though we have been unable to reproduce suppression with complexes formed in vitro over broad ranges of antigen-to-antibody ratios using various purified specific antibodies and appropriate soluble or particulate antigens. Also, heat-aggregated IgGs, which reproduce many of the effects of complexes, did not cause suppression of CTL responses, and storage of sera that favors aggregation of IgGs caused immune sera to lose rather than gain suppressive activity. IgGs are dissociated from antigens and other ligands by acidification; brief acid elution of IgGs from fresh immune sera absorbed on protein A or protein G yielded eluates that were fully suppressive. Acidification, pH 2.0 for 30 min should be more effective in dissociating antigen–antibody complexes, but this procedure also dissociates Ig and TGF-β, which may be the more critical effect of acid treatment. These arguments are supported by recent preliminary observations that indicate that suppression can be mediated by a <300-kD fraction of αSer that would exclude antigen–antibody complexes formed in great antibody excess, but the findings obtained to date do not rule out that suppression can be caused by a >300-kD fraction of αSer.

Our interest in the role of TGF-β originally arose because active TGF-β suppresses activation and proliferation of resting lymphocytes (10–14); glioblastomas and selected other tumors secrete latent TGF-β and are associated with immune suppression (15–17), and an immunogenic murine tumor transfected to secrete latent mTGF-β failed to stimulate CTL in vivo and in vitro (3). Though the experiments presented here do not prove that TGF-β carried by IgG in αSer is responsible for suppression of CTL responses, we think the inference is strong and the concept important enough to be considered until confirmed or refuted.

Ongoing studies are designed to determine whether suppression by αSer is caused by a particular subclass of IgG and whether IgG and TGF-β are secreted as a complex or combine after secretion. Latent TGF-β of ∼110 kD is cleaved to yield the active 25-kD homodimer but the physiological process for activating TGF-β is not understood, though it is accomplished in the laboratory with various proteolytic enzymes (18) and most usually by acidification. This procedure requires pH 2.0 for >15 min; relatively little active TGF-β is obtained at higher pH or during shorter periods of acidification. The inactive portion of latent TGF-β has at least three glycosylation sites that are necessary for latency (19); thus, it is tempting to think that IgG and TGF-β may be linked through carbohydrate chains that are cleaved or digested at target sites, e.g., by Mϕ, causing activation of the carried TGF-β. Possibly the structure of the linkage between TGF-β–IgG in fresh serum may alter on storage or manipulation to account for the lability of suppressive activity of αSer, since the total amount of assayable TGF-β in αSer remains stable during storage.

Since IgGs and latent TGF-β are of comparable molecular mass, only a very small fraction of IgG molecules in αSer must carry TGF-β. However, even a few nanograms of TGF-β per milligram of IgG has the potential for producing important biological effects if TGF-β can be delivered, concentrated, and activated at precise sites. Mϕ focusing the activity of TGF-β delivered by IgG through Fc receptors may explain why TGF-β associated with IgG is an order of magnitude more effective in suppressing CTL responses than free active TGF-β added to cultures, and may account for difficulties using antibodies against TGF-β. Antibodies to TGF-β neutralize only active TGF-β and are effective in bioassays only when they are preincubated with active TGF-β before addition to target cells because of the higher affinity of active TGF-β for receptors than for antibody. Thus, in MLC, antibodies to TGF-β may have different access to TGF-β in the cell–cell interaction or diffusion-limited space between Mϕ

and lymphocytes depending on the form of the antibody. For example, this may explain why a murine mAb against TGF-β1,2,3 (subclass IgG1) does not prevent suppression (data not presented), whereas a murine mAb against TGF-β2,3 (subclass IgG2b) does (Table 3). Possibly the subclass of antibody against TGF-β may be more critical than the epitope on TGF-β.

We have confirmed that the mAb against TGFβ2,3 does not neutralize m or pTGF-β1 in the Mv1Lu bioassay; however, the same antibody does partially neutralize suppression of CTL responses in MLC caused by pTGF-β1 or αSer (Table 3). The different isoforms of TGF-β undoubtedly share epitopes, and receptors for TGF-β are probably not identical on Mv1Lu cells and murine lymphocytes. For these reasons, we do not think it surprising that an antibody that neutralizes the interaction of one isoform of TGF-β with receptors on cells in one system will necessarily be effective in another system and vice versa. Different considerations limit the use of other antibodies against TGF-β in MLC. For example, we cannot use rabbit antibodies because control rabbit IgG is as effective as rabbit anti-TGF-β antibody in preventing suppression by αSer, undoubtedly because rabbit IgG blocks or saturates Fc receptors. Nevertheless, we interpret our overall findings as consistent with the idea that suppression caused by αSer is mediated by TGF-β1.

On a broader scale, the effects of TGF-β are pleiotropic and depend on the type and state of activation or maturation of target cells in many different tissues (20–24), suggesting that the activity of TGF-β must be highly restricted or regulated at different sites. Thus, the idea that the activity of TGF-β in immunity is modulated by Ig suggests one kind of strategy for limiting the activity of TGF-β to the relevant system, just as the association of TGF-β-2 with α-fetal protein (25, 26) suggests a different strategy for limiting activity of TGF-β to the site of maternal–fetal interaction. Thus, IgG localizing TGF-β at antigenic sites could play an important role in the homeostasis of immunity by augmenting proliferation of already activated dominant lymphocyte clones (27), promoting isotype switch (27–29), suppressing activation/proliferation of new specific antigen-reactive clones that may arise during ongoing immunity, and suppressing some autoimmune diseases (30). On the other hand, TGF-β can promote abnormal scarring as well as wound healing (31–37), and stimulate growth of some malignancies (38–41), so that antibody carrying TGF-β to antigen target sites in some autoimmune diseases or cancer may have deleterious effects. In an analogous way, desirable or adverse effects of specific antibodies given passively for experimental or clinical objectives (42) may depend on the presence and nature of TGF-β carried by IgG. Thus, the finding that IgG can carry biologically effective TGF-β has many important implications.

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