A Transforming Growth Factor β-like Immunosuppressive Factor in Immunoglobulin G-binding Factor

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Summary

Immunoglobulin G-binding factors (IgG-BF), which are produced by cells of the immune system, inhibit antibody production. In this paper, we show that transforming growth factor-β (TGF-β) suppresses secondary in vitro anti–sheep red blood cell responses of mouse splenocytes and lipopolysaccharide- or anti-IgM–stimulated mouse B cell responses in a way similar to, and with the same kinetics as, rodent IgG-BF. Moreover, the immunosuppressive activity of IgG-BF was totally neutralized by polyclonal and monoclonal anti–TGF-β antibodies and it eluted with TGF-β by gel exclusion chromatography, suggesting that a TGF-β–like immunosuppressive factor is present in IgG-BF. We also show that TGF-β behaves as an IgG-BF since it binds to insolubilized IgG, but not to insolubilized F(ab')₂ or bovine serum albumin. Altogether, the data support the concept of a biological role for TGF-β in the IgG-mediated negative feedback of antibody responses.

Antibody production is the consequence of antigen-triggered B cell activation, proliferation, and differentiation into plasma cells. Each step of this complex process is closely regulated by enhancing or inhibiting factors. Cytokines such as IL-4, IL-2, or IL-5 drive B cells through the cell cycle, and IFN-γ, TGF-β, or IL-4 modulate the isotypic profile of the antibody responses (1, 2). Among the regulatory loops involved in antibody production, a negative feedback is exerted by IgG antibodies. It requires the Fc portion of the IgG and decreases the production of all Ig isotypes. Two nonexclusive mechanisms have been proposed for this activity. First, the cross-linking of the B cell receptor to receptors for the Fc portion of IgG (FcγR)[1] at the cell membrane, via IgG antibodies reacting both with B cell receptor and with FcγR, induces a negative signal in B cells by inhibiting the early events of activation (3–5). Second, the interaction of IgG immune complexes with FcγR positive cells induces the production of suppressor factors (6). Among these factors, IgG-binding factors (IgG-BF) (7) have been shown to inhibit both IgM and IgG antibody production (8–13).

The origin and nature of IgG-BF have been extensively investigated. IgG-BF are produced by activated T and B lymphocytes, NK cells, macrophages, and cells of myelomonocytic origin (7–9, 12–14). Since these cells express

FcγR and since IgG increases FcγR expression and IgG-BF production (9, 14), the soluble forms of FcγR (sFcγR) were thought to be likely candidates for suppressor IgG-BF.

Three types of FcγR have been identified (15): FcγRI is a high affinity receptor for monomeric IgG, while FcγRII and FcγRIII are low affinity receptors that bind IgG immune complexes. In mice, the ectodomains of FcγRII and FcγRIII exhibit 95% sequence homology. Soluble forms of FcγR are generated either by splicing of the exon encoding the transmembrane region of FcγRII (16) or by proteolytic cleavage of the extracellular part of the membrane FcγRII (17) or FcγRIII (18). To establish whether sFcγR are at least partially responsible for the suppressive activity of IgG-BF, a recombinant murine sFcγR corresponding to the FcγRII ectodomain, and thus highly homologous to membrane-cleaved FcγRII and FcγRIII, was produced by transfection of eukaryotic cell lines with a mutant complementary DNA containing a stop codon (19). This sFcγR was purified up to 98% homogeneity by a succession of chromatography steps, including affinity chromatography on insolubilized IgG (19, 20). The resulting preparations inhibited IgM and IgG antibody production to SRBC both in vitro (18–21) and in vivo (18, 21).

Cells of the immune system produce endogenous immunoregulatory molecules such as TGF-β. TGF-β belongs to a superfamily of structurally related proteins and is produced by a variety of cells, including fibroblasts, activated lymphocytes, macrophages, and platelets (22). The three mammalian isoforms of TGF-β (TGF-β1, -β2, and -β3) share 70–80% amino acid sequence identity and each iso-

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form is highly conserved (22). In addition to its initially described activity as transforming factor, TGF-β exerts pleiotropic activities, regulating many immune and inflammatory processes such as cell proliferation (22, 23), antibody production (24, 25), and cytotoxic T (26) and LAK cell generation (27, 28). TGF-β is synthesized and secreted as a latent molecule that does not bind to the TGF-β receptors. Various conditions, such as acid or alkaline treatment, heating at 100°C, as well as proteases and glycosidases, can activate latent TGF-β into a biologically active molecule (29). An intriguing property of TGF-β family members is that, in addition to their cellular receptors, they bind to several proteins such as latency-associated peptide (30), α2-macrocroglobulin (31), α-fetoprotein (32), fibronectin (33), sulphate proteoglycan decorin (34), and mannose 6-phosphate receptor (35).

Since cells that are used to produce sFcγR also produce TGF-β, and since TGF-β has a high anti-proliferative activity, the present study was conducted to provide a better understanding of the respective roles of TGF-β and sFcγR in the suppressive activity of IgG-BF. Soluble FcγR secreted by transfected cell lines and by mouse activated T cells was used as the source of IgG-BF. First, we showed that TGF-β was present, together with sFcγR, in affinity-purified IgG-BF. Second, we observed that IgG-BF and purified porcine TGF-β inhibit B cell proliferation and antibody production with identical kinetics. Third, we demonstrated that neutralizing antibodies to TGF-β abolished the suppressive activity of IgG-BF and that the active factor eluted with TGF-β by gel exclusion chromatography, and not with sFcγR. Finally, we showed that purified TGF-β binds to IgG-Sepharose, and not to F(ab')₂- or BSA-Sepharose, thus behaving as an IgG-BF. This property suggests a biological role for TGF-β, together with IgG antibodies, in the IgG-mediated negative feedback of antibody responses.

Materials and Methods

Mice. 8–10-wk-old (C57BL/6J × DBA/2J)F1 (B6D2)F1 male mice were used for the preparation of resting B cells. 4-wk-old C3H mice were used as donors of thymus cells and DBA/2 mice, 8-wk-old, were used as recipients for the preparation of alloantigen-activated T cells. All mice were obtained from IFFA CREDO (L'arbresle, France).

Antibodies and Growth Factors. The rat hybridoma cell line JIj.10-producing IgM anti–mouse Thy-1 mAb was obtained from the American Type Culture Collection (Rockville, MD). The rat anti–mouse FcγRII/III mAb 2.4G2 (36) was purified by chromatography on protein G. The neutralizing polyclonal chicken IgY anti–porcine or anti–human TGF-β1 and control IgY were purchased from R & D Systems, Inc. (Minneapolis, MN). Monoclonal mouse IgG₁ anti–TGF-β1, -β2, and -β3 (generated against bovine TGF-β2) was obtained from Genzyme (Cambridge, MA). Mouse IgG₂ₐ and IgG₁ were purified by chromatography on protein A–Sepharose of culture supernatant of the IgG₂ₐ anti-SRBC and IgG₁ secreting hybridoma line UN2C3 and U182, respectively (19). F(ab')₂ fragments of rabbit IgG (Nordic, Tilburg, The Netherlands) were obtained by pepsin digestion (2% wt/wt)

followed by protein A–Sepharose chromatography. The IgG and F(ab')₂ fragments were pure as determined by Coomassie blue staining after SDS-PAGE. They were coupled to activated Sepharose 4B (7 mg protein/ml Sepharose) according to the manufacturer's recommendations (Pharmacia, St. Quentin en Yvelines, France). To obtain polyclonal anti–IgG-BF antibodies, rabbits were hyperimmunized by a footpad injection of 100 μg of IgG-BF isolated as described below in CFA, followed by three successive boostings by intraperitoneal injections at 2-wk intervals. Goat affinity-purified F(ab')₂ fragments to murine IgM were purchased from Organon Teknica Corp. (Durham, NC). TGF-β1 purified from porcine platelets was purchased from R & D Systems. Before use, the lyophilized preparations were dissolved in 4 mM HCl containing 1 mg/ml BSA. In some experiments, 1 ml porcine TGF-β1 (200 ng) in 20 mM Tris buffer, pH 7.6, was incubated with 0.1 ml Sepharose-coupled rabbit IgG or F(ab')₂ fragments of rabbit IgG for 18 h at 4°C. After five washes in the same buffer, bound material was eluted in 0.3 ml 0.2 M glycine HCl buffer, pH 2.8, neutralized and dialyzed against PBS before use. LPS from *Escherichia coli* was purchased from Sigma Immunochemicals (St. Louis, MO).

Purification of IgG-BF Produced by Transfected Cell Lines. Fibroblastic mouse L and baby hamster kidney (BHK) cell lines were transfected with pKC3 plasmid containing a cDNA encoding the 174-NH₂-terminal amino acids of mouse FcγRII (19, 20). Three cell lines, Cu1B3 derived from L cells, and IVC2 and 6/9CII derived from BHK cells, were obtained. To scale up production, these transfected cell lines were grown on hollow fibers in a perfusion cell culture system (AcusystJr.; Endotronics Inc., Coon Rapids, MN). As estimated by ELISA, Cu1B3, IVC2, and 6/9CII cell lines produced 1, 5.6, and 5.2 mg/d sFcγR, respectively (20). Recombinant sFcγF was purified from culture medium by ion exchange chromatography followed by affinity chromatography. The culture medium (150 ml) was equilibrated in 20 mM Tris buffer, pH 7.6. All equilibrations steps were performed by Sephadex G25 (Pharmacia) chromatography. The material was then applied on a column containing 10 ml S-Sepharose (Fast Flow; Pharmacia). After washes with the same buffer, proteins were eluted with 20 mM Tris buffer, pH 7.6, containing 0.5 M NaCl. After equilibration in 20 mM Tris buffer, pH 7.6, the material from BHK and L transfected cell lines was applied to 1 ml of rabbit IgG- or mouse IgG₂ₐ-coupled Sepharose, respectively. The column was washed with equilibration buffer and the proteins were eluted with 0.1 M sodium acetate buffer, pH 4. The eluted material was equilibrated in PBS, spun for 15 min at 9000 g, sterilized by UV irradiation, and stored at −80°C in aliquots. These procedures led to the isolation of IgG-BF containing 98% recombinant sFcγR with a 30% yield (20). Deglycosylation of 20 μg purified material was performed by incubation with 200 U N-glycosidase F (Biolabs, Beverly, MA). For gel exclusion chromatography, 0.25 ml of IgG-BF was applied at 0.5 ml/min on a Superdex 75 HR10/30 column (Pharmacia) equilibrated in PBS. Fractions (0.5-ml) were collected and tested for biological activity and TGF-β and sFcγR contents.

Purification of IgG-BF from Supernatants of Alloantigen-activated T Cells (ATC). ATC were obtained as previously described (6). Briefly, 10⁸ thymocytes from C3H mice were injected in the tail vein of 850-rad irradiated DBA/2 recipients. 5 d later, spleen cells (95% Thy-1⁺) were harvested and incubated for 2 h at 37°C in HBSS (GIBCO BRL, Paisley, Scotland). Supernatants were collected and spun at 1800 g. IgG-BF was purified by affinity chromatography on rabbit IgG as described (7, 8).

B Cell Responses. Spleen cell suspensions were depleted of T

cells by treatment with crude supernatant of JIj.10 cell line for 30 min at room temperature in HBSS (Gibco) followed by two successive 15-min incubations at 37°C with the appropriate dilution (previously determined) of rabbit C (serum from 3-wk-old rabbit). Resting B cells were isolated by Percoll (Pharmacia) density centrifugation as previously described (24). The cells banding between 70 and 60% Percoll were collected and washed twice with HBSS. These cells, representing the source of resting B lymphocytes, were 95% IgM$^+$ and did not proliferate significantly in cultures performed in the presence of 2 μg/ml Con A (Sigma; data not shown). Resting B cells (5 × 10^4) were cultured (in duplicate) in 0.2 ml of RPMI 1640 culture medium (GIBCO) supplemented with 2 mM L-glutamine (GIBCO), 100 U/ml of penicillin and 100 μg/ml of streptomycin (GIBCO), 5 × 10^{-5} M β-mercaptoethanol (GIBCO), and 10% heat-inactivated FCS (Seromed, Berlin, Germany), containing 50 μg/ml of LPS or 15 μg/ml of F(ab')$_2$ anti-IgM at 37°C, in an atmosphere containing 5% CO$_2$ in 96-well microplates (Becton Dickinson, Grenoble, France). 48 and 66 h after the onset of anti-IgM stimulation and LPS stimulation, respectively, proliferation was assayed by pulsing the cells with 0.5 μCi per well of [^{3}H]thymidine (Amersham, Les Ulis, France) for the last 18 h of culture. Cells were then harvested with a 96-well harvester (Skatron Instruments, Norway), and collected on a glass fiber filter (Pharmacia). The radioactivity was counted in a Betaplate (Pharmacia LKB). 7 d after initiation of the cultures stimulated by LPS, the supernatants were collected by centrifugation and tested for their Ig content.

ELISA. The amounts of Igs were determined by an isotype-specific ELISA (24) using 96-well flat-bottom immunoplates (DK 4000; Nunc, Roskilde, Denmark) coated with goat antibodies directed against mouse IgM or IgG$_3$ (Southern Biotechnology Associates, Birmingham, AL) and alkaline phosphatase-labeled goat anti–mouse IgM or IgG$_3$ antibodies (Southern Biotechnology) for detection. Ig concentrations were calculated from the absorbance units by extrapolation from standard curves determined with purified mouse antibodies (Southern Biotechnology). Titration of sFcγR was performed as previously described (20). Briefly, 96-well flat-bottom immunoplates (Nunc) were coated with 2.4G2 mAb, washed, and saturated. 50 μl of serial twofold dilutions of sFcγR-containing samples or 98% pure recombinant sFcγR used as a standard were added and incubated for 2 h at 20°C. Plates were then incubated with rabbit IgG anti–IgG-BF (10 μg/ml). After washes, goat anti–rabbit IgG coupled to alkaline phosphatase (Southern Biotechnology) was added for the detection. Optical densities were measured at 405 nm with an ELISA reader (Titertek Multiskan; Labsystems France, Les Ulis, France) connected to an Olivetti M240 computer. The sensitivity of this ELISA is 25 ng/ml. An ELISA for TGF-β has been developed in our laboratory. The 96-well flat-bottom immunoplates (Nunc) were coated overnight at 4°C with mouse monoclonal anti–TGF-β diluted (5 μg/ml) in 0.05 M Na$_2$CO$_3$/NaHCO$_3$, pH 9.6. After washes and saturation with 0.05% PBS, Tween 20 (Merck, Schuchardt, Germany), and 2% BSA (Sigma) for 1 h at 37°C, 50 μl of serial twofold dilutions in 0.05% PBS, Tween 20, and 2% BSA of samples acidified as described (27) were incubated for 1 h at 37°C. After washes, chicken IgY anti–human TGF-β1 antibodies diluted (1:500) in PBS 0.05%, Tween 20, and 2% BSA were added and incubated overnight at 4°C. For detection, plates were first incubated with goat anti–chicken IgY coupled to alkaline phosphatase (Southern Biotechnology), 1:10,000, 3 h and 30 min at 20°C, and then with the substrate (p-nitrophenyl phosphate; GIBCO). After 15 min, the amplifier was added (GIBCO) and optical densities were measured at 492 nm. Human rTGF-β1

was used as a standard (R & D Systems). This ELISA detects concentrations of human TGF-β1 that are >1 ng/ml. Its reactivity for TGF-β2 and -β3 is unknown at the present time.

Secondary In Vitro Antibody Responses. Spleen cells (6 × 10^6) from B6D2 mice primed in vivo with SRBC were cultured in 1 ml RPMI, 10% FCS, and 1% horse serum for 5 d with SRBC as described (37). Indirect IgG plaque-forming cells (PFC) were enumerated by an indirect hemolytic plaque assay in liquid medium using rabbit anti–mouse IgG antiserum and guinea pig serum as a source of complement. Products or PBS, used as a positive control, were added on day 0 in a final volume of 0.1 ml. Results are expressed as the number of indirect PFC per 10^6 recovered cells.

SDS-PAGE and Western Blotting. One-dimensional discontinuous SDS-PAGE was carried out on 10 or 12.5% polyacrylamide gels. Before layering onto the gel, proteins were boiled for 3 min in 50 mM Tris-HCl, pH 6.8, 1% SDS, 10% glycerol, and 0.001% bromophenol blue. For reducing conditions, 0.7 M β-2 mercaptoethanol (Sigma) was added. For Western blotting, nonreduced samples were run on 12.5% acrylamide SDS minigels and transferred to nitrocellulose filters (Schleicher & Schuell, Darsel, Germany) using a semi-dry transfer apparatus (Bio Rad Laboratories, Hercules, CA). Electroblotting was performed at 22 V using 25 mM Tris, 192 mM glycine, and 20% ethanol as transfer buffer. The nitrocellulose membranes were saturated for 1 h at 37°C in TBS (10 mM Tris, 150 mM NaCl, pH 7.4) containing 5% BSA (Sigma). Primary antibodies (chicken anti–porcine TGF-β1 or rabbit anti–IgG-BF) diluted in TBS with 5% BSA (wt/vol) (3 μg/ml for anti–TGF-β1, 1:1,000 for anti–IgG-BF) were incubated with the blocked nitrocellulose membranes overnight at 4°C. Excess antibody was removed with three 10-min washes in TBS. The nitrocellulose filters were then incubated with secondary antibodies coupled to alkaline phosphatase (Southern Biotechnology) diluted to 1:1,000 in TBS with 5% BSA (wt/vol) for 1 h at room temperature. After five 10-min washes in TBS, filters were revealed using the BCIP/NBT Phosphatase Substrate System (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

Results

Soluble FcγR and TGF-β Are Present in IgG-BF. Culture medium of the Cu1B3 cell line transfected with a cDNA encoding sFcγR was subjected to ion exchange chromatography and affinity chromatography on Sepharose-coupled IgG. The suppressive activity of the preparations was assessed in three separate experiments, by inhibition of a secondary in vitro anti-SRBC response, evaluated by counting the indirect IgG-PFC. The starting material and the affinity-purified preparation, i.e., IgG-BF, inhibited antibody production to SRBC. A >10 fold-enrichment in specific activity was achieved by the purification process, since 50% inhibition was obtained with 2.75 μg/ml of affinity-purified IgG-BF as opposed to 30 μg/ml of starting material (Fig. 1 *A*).

SDS-PAGE analysis of the proteins present in starting material and in IgG-BF purified from supernatants of Cu1B3 cell line revealed enrichment for a group of proteins ranging from 30 to 45 kD (Fig. 1 *B*). Quantification by ELISA showed that this enrichment was 300-fold. As previously observed (16, 17), these proteins gave a single band corresponding to the 20-kD polypeptide of sFcγR after deglycosylation (data not shown). When the preparation was ana-
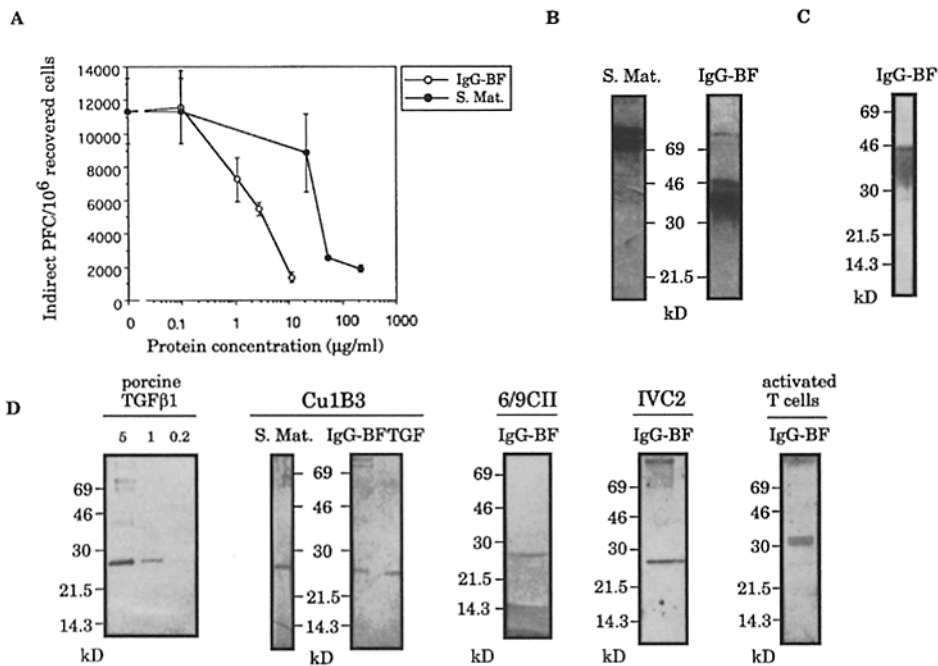
A   B   C

D

Figure 1. Soluble FcγR and TGF-β are present in different IgG-BF. (A) Inhibition of secondary in vitro anti-SRBC response by starting material (S. Mat) and IgG-BF from CuIB3 cell line. Products were added on day 0, and the response was measured on day 5. Results are expressed as the number of indirect PFC per 10⁶ recovered cells (means ± SEM of duplicate values). (B) Analysis of S. Mat. and IgG-BF from the CuIB3 cell line by SDS-PAGE followed by silver staining (5 μg per sample). (C) Analysis of IgG-BF (25 μg) from CuIB3 cell line by Western blotting with rabbit anti-IgG-BF antiserum (1:1,000). (D) Analysis of porcine TGF-β1 (5, 1, and 0.2 ng), S. Mat. (25 μg) from the CuIB3 cell line, IgG-BF purified from culture medium of CuIB3 (25 μg), 6/9CII (17.5 μg), and IVC2 (25 μg) cell lines or from activated T cells (150 ng) by Western blotting with chicken anti–porcine TGF-β1 (3 μg/ml) antibodies.

lyzed by Western blotting using an antiserum to IgG-BF, a major band corresponding to sFcγR (30–45 kD) was visible (Fig. 1 C). When analyzed using anti–TGF-β antibodies, one band corresponding to a 25 kD-polypeptide was visible in addition to slower migrating ones (Fig. 1 D). As estimated by Western blotting by comparison with different doses of standard porcine TGF-β, 0.5 ng 25 kD TGF-β was detected in 30 μg of purified IgG-BF. Similar results were found with another IgG-BF preparation from the same cell line (data not shown). As illustrated in Fig. 1 D, 25 kD TGF-β was also detected by Western blotting in IgG-BF purified from the culture media of other cell lines secreting recombinant sFcγR, such as 6/9CII (four preparations) and IVC2 (five preparations). In IgG-BF from the IVC2 cell line, anti–TGF-β antibodies revealed additional slower migrating proteins (Fig. 1 D).

Activated T cells secrete soluble FcγR (7, 38) as well as TGF-β (38). IgG-BF was purified from culture supernatants of mouse ATC and analyzed similarly. As shown in Fig. 1 D, the T cell–produced IgG-BF contained 25 kD TGF-β in addition to slower migrating TGF-β–reactive components including a prominent protein doublet.

Comparative Inhibitions of IgG-BF and TGF-β on B Cell Responses.   Antibody production to SRBC is a complex process that involves different cell types. To more clearly define the experimental model, we used purified mouse resting splenic B cells directly stimulated either by anti-IgM F(ab')₂ antibodies (15 μg/ml) or by LPS (50 μg/ml). Anti-IgM stimulation triggered B cell proliferation but not Ig production (Fig. 2), whereas LPS activation drove B cells through proliferation to IgM and IgG₃ production (Fig. 3). On day 0, IgG-BF or porcine TGF-β1 was added to cell
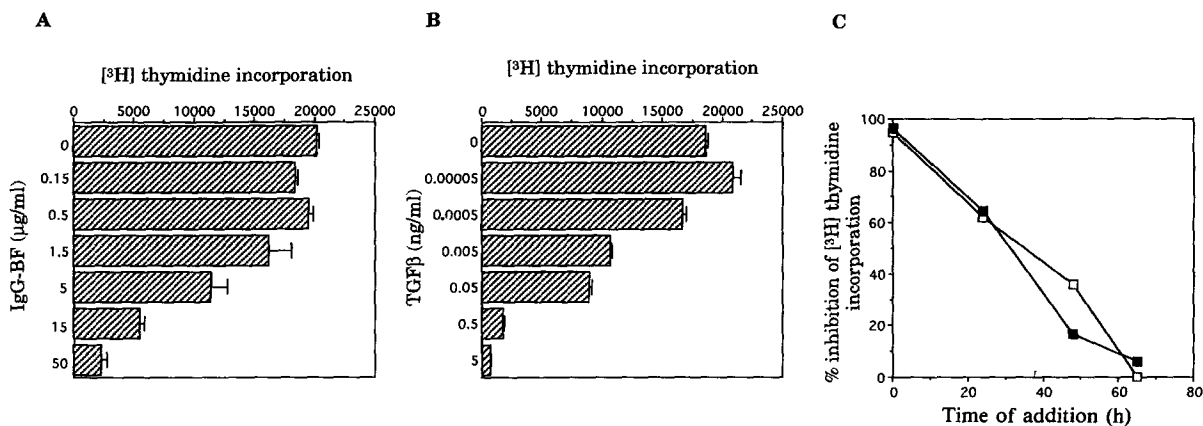
A   B   C

Figure 2. Immunosuppressive activities of IgG-BF and of TGF-β on B cell responses stimulated by anti-IgM. Various doses of IgG-BF (A) or of porcine TGF-β1 (B) were added on day 0. Cell proliferation was measured on day 3 by [³H]thymidine incorporation. The results are representative of five experiments. Each bar represents the mean ± SEM of duplicate samples. In a single kinetic experiment (C), IgG-BF (50 μg/ml) or porcine TGF-β1 (0.5 ng/ml) was added at various times after the onset of cultures, and cell proliferation was measured on day 3. —□—, IgG-BF; —■—, TGF-β.

A
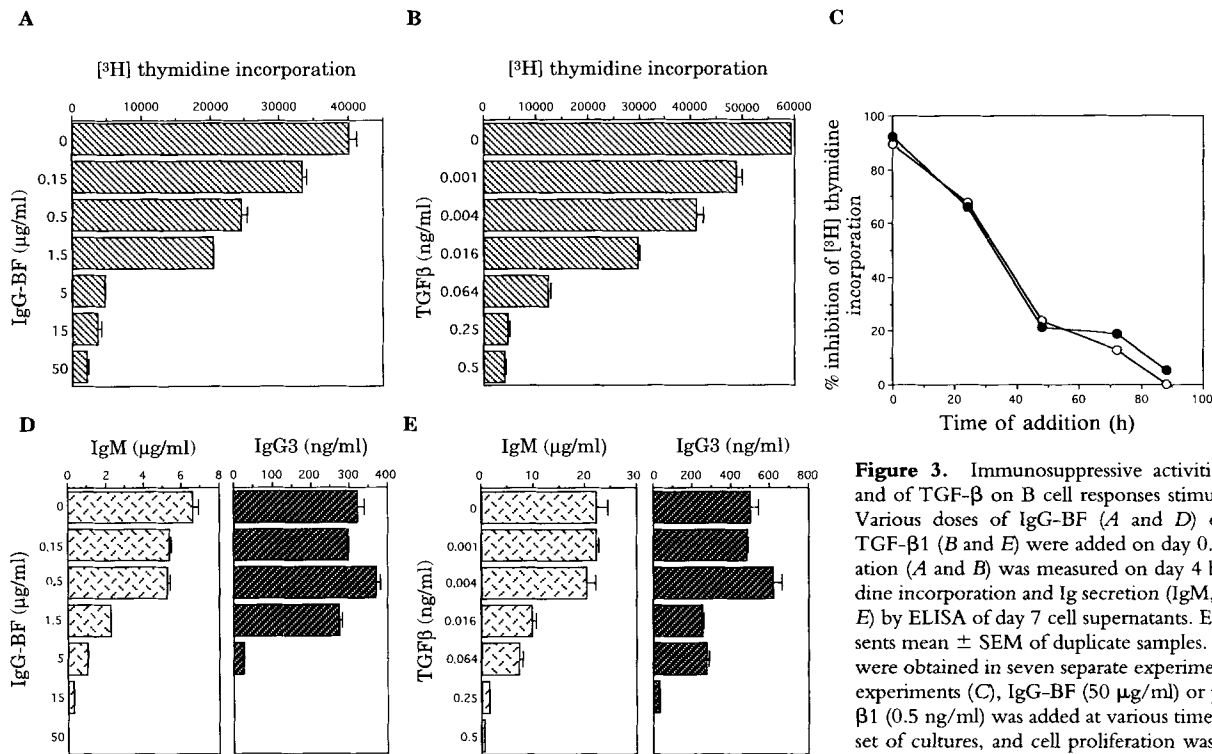
[³H] thymidine incorporation

0   10000   20000   30000   40000

IgG-BF (μg/ml)
0
0.15
0.5
1.5
5
15
50

B

[³H] thymidine incorporation

0   10000   20000   30000   40000   50000   60000

TGFβ (ng/ml)
0
0.001
0.004
0.016
0.064
0.25
0.5

C

% inhibition of [³H] thymidine incorporation
100
80
60
40
20
0

0   20   40   60   80   100
Time of addition (h)

D

IgM (μg/ml)   IgG3 (ng/ml)
0 2 4 6 8 0 100 200 300 400

IgG-BF (μg/ml)
0
0.15
0.5
1.5
5
15
50

E

IgM (μg/ml)   IgG3 (ng/ml)
0   10   20   30 0 200 400 600 800

TGFβ (ng/ml)
0
0.001
0.004
0.016
0.064
0.25
0.5

Figure 3. Immunosuppressive activities of IgG-BF and of TGF-β on B cell responses stimulated by LPS. Various doses of IgG-BF (*A* and *D*) or of porcine TGF-β1 (*B* and *E*) were added on day 0. Cell proliferation (*A* and *B*) was measured on day 4 by [³H]thymidine incorporation and Ig secretion (IgM, IgG₃) (*D* and *E*) by ELISA of day 7 cell supernatants. Each bar represents mean ± SEM of duplicate samples. Similar results were obtained in seven separate experiments. In kinetic experiments (*C*), IgG-BF (50 μg/ml) or porcine TGF-β1 (0.5 ng/ml) was added at various times after the onset of cultures, and cell proliferation was measured on day 4. —O— , IgG-BF; —●— , TGF-β. The results are representative of two independent experiments.

cultures. Figs. 2 and 3 illustrate representative results from a dozen independent experiments using IgG-BF from either Cu1B3 (two and four experiments performed with anti-IgM and LPS, respectively) or 6/9CII (three experiments for each of the two stimuli) cell lines and from four independent experiments using porcine TGF-β1.

Addition of increasing doses of affinity-purified IgG-BF or porcine TGF-β1 resulted in a dose-dependent inhibition of B cell proliferation induced by stimulation by anti-IgM (Fig. 2, *A* and *B*) or LPS (Fig. 3, *A* and *B*). In the latter case, total inhibition of IgM and IgG₃ production was also observed in IgG-BF– and in TGF-β–treated cultures (Fig. 3, *D* and *E*). A 50% inhibition of proliferation and antibody production was achieved by 1.5 and 5 μg/ml of IgG-BF, and 0.016 and 0.05 ng/ml of porcine TGF-β1 in the LPS- and anti-IgM–stimulated cultures respectively, which is compatible with the hypothesis that the TGF-β present in the IgG-BF preparations might be responsible for suppression. This was reinforced by three independent kinetic experiments in which porcine TGF-β1, at a dose of 0.05 ng/ml, or IgG-BF, at a dose of 50 μg/ml, was added at different times to B cells stimulated by anti-IgM antibodies (Fig. 2 *C*) or LPS (Fig. 3 *C*). These figures illustrate the result of one of the three experiments performed. Cell proliferation, as assessed by [³H]thymidine incorporation on day 3 for anti-IgM stimulation or on day 4 for LPS stimulation, was inhibited with the same kinetics by the two preparations, with a maximum when added at the onset of the cultures, and no significant inhibition when added during the last 18 h of culture.

Effect of Anti–TGF-β Antibodies on the Inhibitory Activity of IgG-BF. To investigate the role of TGF-β in the suppressive activity exerted by IgG-BF, neutralizing anti–TGF-β antibodies were added in four repeated independent experiments to cultures of resting B lymphocytes treated with IgG-BF from the 6/9CII cell line or with porcine TGF-β1. As illustrated in Fig. 4, the addition of increasing doses of chicken anti–porcine TGF-β1 to purified B cells stimulated with anti-IgM antibodies (Fig. 4 *A*) or with LPS (Fig. 4 *B*) increased the proliferative response by ~20%, suggesting that TGF-β might be produced during culture and neutralized by the antibodies cross-reacting with endogenous mouse TGF-β. Addition of control chicken IgY had no effect as well as addition of rat anti–mouse FcγR 2.4G2 antibodies (35; data not shown). The addition of 15 μg/ml IgG-BF or 0.1 ng/ml porcine TGF-β1 resulted in ~80% inhibition of B cell proliferation, which was completely reversed, in both cases, by the addition of anti–TGF-β, but not by control IgY or 2.4G2 antibodies (Fig. 4, *A* and *B*; data not shown). To ascertain whether the removal of inhibitory activity was indeed caused by anti–TGF-β antibodies, a mouse mAb was used to neutralize TGF-β activity. It fully reversed the suppressive activity exerted by IgG-BF on LPS-induced B cell proliferation, as well as that of porcine TGF-β1, although less efficiently. An isotype-matched IgG₁ had no effect (Fig. 4 *C*).

To establish whether TGF-β was also involved in the suppression exerted by IgG-BF on antibody production to SRBC, chicken polyclonal antibodies or mouse mAbs to TGF-β were added to splenocytes that had been stimulated
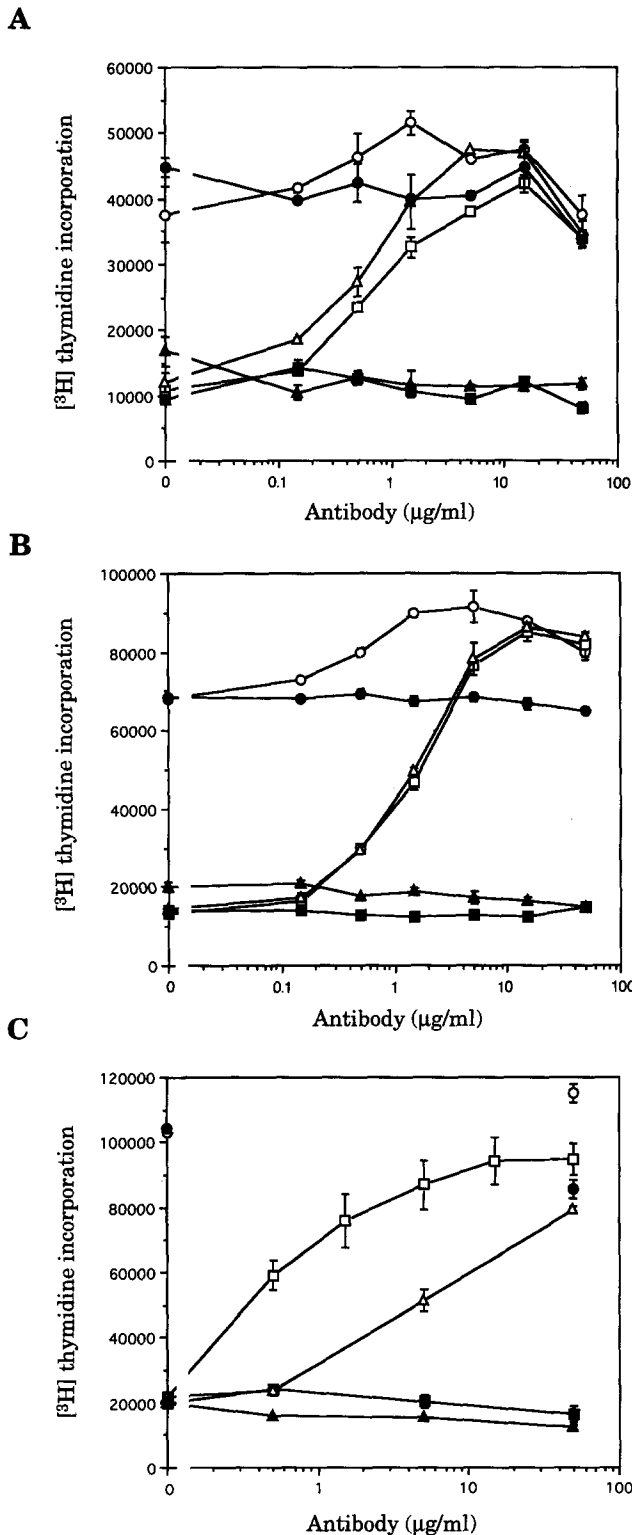
A

B

C

Figure 4. Anti–TGF-β neutralized the antiproliferative activities of IgG-BF and TGF-β. IgG-BF (50 μg/ml), porcine TGF-β1 (1 ng/ml), or control medium was added on day 0 to anti-IgM– (*A*) or LPS– (*B* and *C*) stimulated B cell cultures. Various concentrations of chicken polyclonal anti–porcine TGF-β1 antibodies or control IgY (*A* and *B*), and of mouse monoclonal antibovine TGF-β or control mouse IgG₁ (*C*) were added on day 0 to these cultures. Proliferative responses were measured on day 3 for anti-IgM–stimulated cultures and on day 4 for LPS–stimulated cul-

in vitro with SRBC in the presence of 15 μg/ml IgG-BF or 3 ng/ml TGF-β. We showed in two independent experiments that the inhibitory activity exerted by both factors on the production of IgG-PFC was reversed by 45 μg/ml polyclonal (Fig. 5 *A*) or 30 μg/ml monoclonal (Fig. 5 *B*) anti–TGF-β antibodies, whereas isotype-matched control Ig had no effect (data not shown). As for mouse B cells, the addition of anti–TGF-β antibodies increased the number of PFC by ~20% (Fig. 5, *A* and *B*), suggesting that TGF-β produced during the course of an antigen-stimulated immune reaction modulates the antibody response.

Separation of TGF-β and sFcγR by Gel Exclusion Chromatography. The inhibition of the suppressive activity of IgG-BF by anti–TGF-β antibodies could be caused by the neutralization of TGF-β present in the IgG-BF preparations or to TGF-β induced during culture. We therefore attempted to separate TGF-β from sFcγR before addition to the cultures. This was achieved on a Superdex 75 column (Pharmacia). Fig. 6 represents an illustrative experiment, out of three, in which 91% of TGF-β, as revealed by Western blotting, eluted in the void volume, whereas sFcγR, as revealed by ELISA with anti-FcγR antibodies, eluted at $V_e/V_o = 1.3$ (11.5 ml). The suppressive activity on B cell proliferation superimposed with the bulk of TGF-β, with no detectable activity in the fractions containing sFcγR, demonstrating that exogenous TGF-β was responsible for most, if not all, the inhibition of B cell responses.

Binding of TGF-β to IgG. Since TGF-β was present in IgG-BF isolated by affinity chromatography with insolubilized IgG, binding of purified porcine TGF-β1 to IgG was directly assessed on Sepharose beads coupled with rabbit IgG or F(ab')₂ fragments of rabbit IgG as controls. TGF-β was detected by Western blotting, ELISA, and by measuring its antiproliferative activity on LPS-stimulated B cell responses and on secondary anti-SRBC IgG response. As illustrated in Fig. 7 *A*, TGF-β was recovered in the acid eluates of IgG columns, but was barely detectable in those from F(ab')₂ columns. During the course of three independent experiments, 28.6 ± 8% of the applied material was present in the IgG eluates and only 2 ± 1.3% in the F(ab')₂ eluates, as estimated by Western blotting. The results were confirmed by ELISA (data not shown). As shown in Fig. 7 *B*, TGF-β inhibited, in a dose-dependent fashion, the proliferation of mouse resting B cells stimulated by LPS. The comparison of the antiproliferative activities, in two independent experiments, of the various fractions showed that the eluates from IgG immunoadsorbents and the effluents from F(ab')₂-Sepharose were almost as suppressive as the starting material, whereas the effluents of the IgG-immunoadsorbents and the eluates from F(ab')₂-Sepharose were 20-fold less active (Fig. 7 *B*). When added to splenocyte cultures stimulated with SRBC, TGF-β inhibited IgG an-
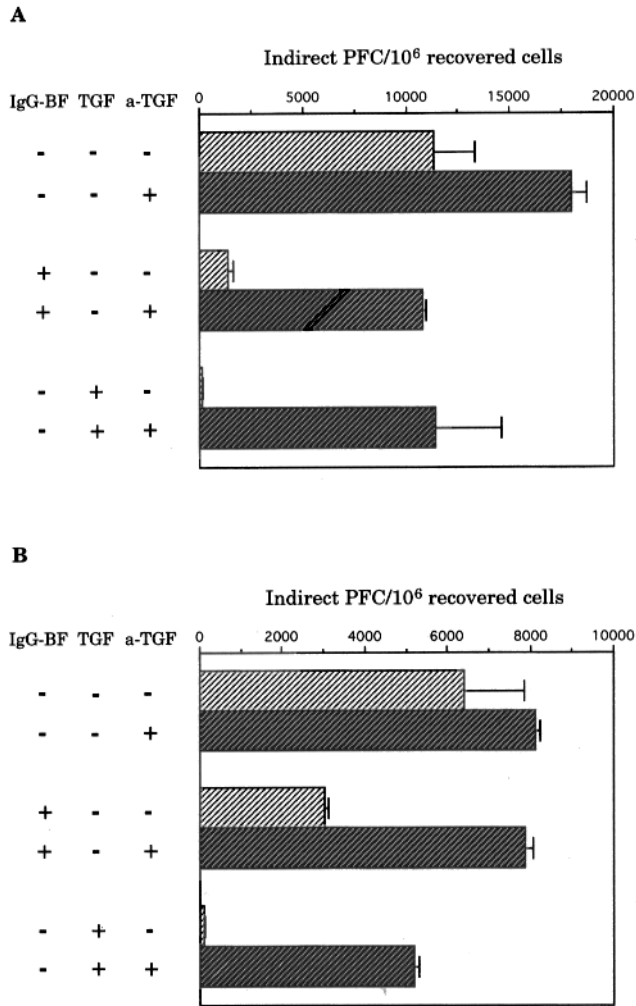
tures. Each point represents the mean ± SEM of duplicate samples. These results are representative of four separate experiments. —O— , anti-TGF/control; —□— , anti-TGF/IgG-BF; —△— , anti-TGF/TGF; —●— , IgY/control; —■— , IgY/IgG-BF; —▲— , IgY/TGF.

A

Indirect PFC/10⁶ recovered cells

B

Indirect PFC/10⁶ recovered cells

Figure 5. Anti–TGF-β reversed the inhibitory activities of IgG–BF and TGF-β on secondary anti–SRBC responses in vitro. IgG–BF (5.5 μg) or porcine TGF-β1 (3 ng) was added to anti–SRBC spleen cell cultures in the presence or in the absence of chicken polyclonal anti–porcine TGF-β1 (45 μg) (A) or mouse monoclonal antibovine TGF-β (30 μg) (B). Results representative of two independent experiments are expressed as the number of indirect PFC per 10⁶ recovered cells (means ± SEM of duplicate values).

tibody production. The suppressive activity was retained, and could be eluted from, IgG but not F(ab')₂ immunoadsorbents (Fig. 7 C). Overall, these results show that active TGF-β binds to insolubilized IgG via its Fc portion and can be eluted from it in an active form.

Discussion

The studies reported here demonstrate that a TGF-β–like immunosuppressive factor is responsible for the inhibition of B cell proliferation and antibody production exerted by IgG–BF preparations. Several lines of evidence lead to this conclusion. (a) TGF-β molecules can be detected in IgG–BF preparations; (b) not only do TGF-β and IgG–BF inhibit B cell proliferation and Ig production in a similar way, but they do so with identical kinetics; and (c) the suppres-
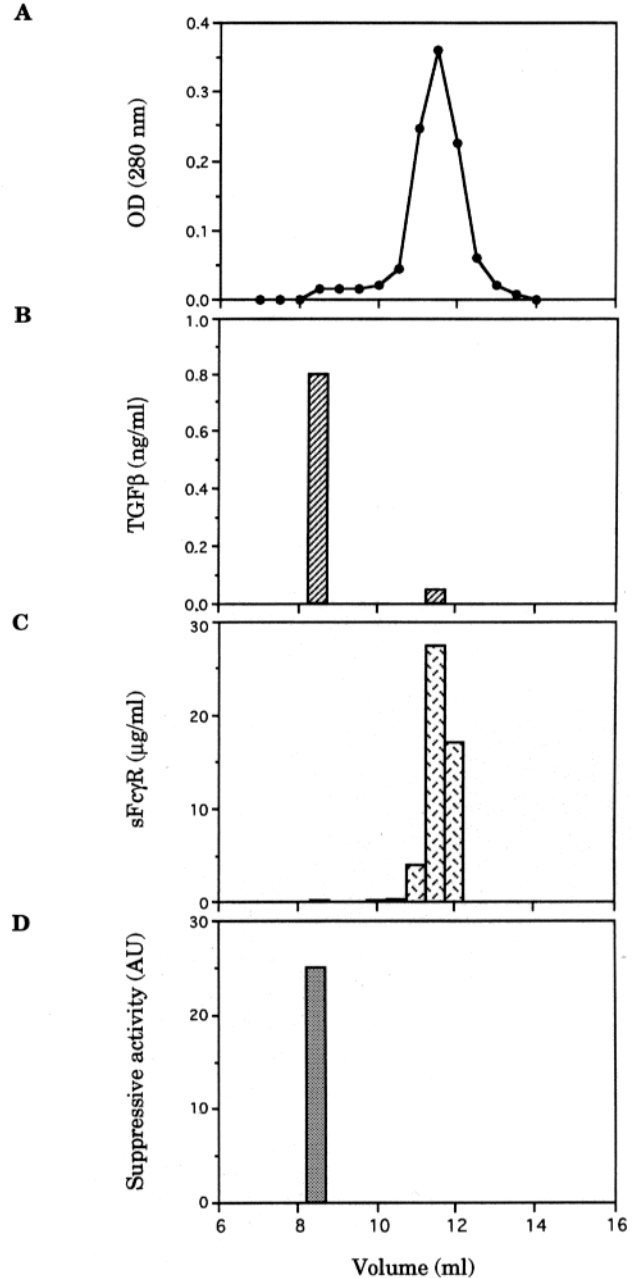

Figure 6. Separation of TGF-β from sFcγR by gel exclusion chromatography. (A) OD-280 nm; (B) TGF-β concentration; (C) sFcγR concentration; (D) antiproliferative activity on LPS-induced B cells (AU, arbitrary units). Similar results were obtained in three separate experiments.

sive activity exerted by IgG–BF is totally reversed by polyclonal and monoclonal antibodies to TGF-β.

In mammals, three isoforms of TGF-β share ∼70–80% homology and are almost completely conserved between species (22). The fact that the suppressive activity of IgG–BF preparations on LPS- or anti-IgM–stimulated B cells, as well as on anti-SRBC responses, is inhibited by polyclonal and monoclonal anti–TGF-β antibodies demonstrates that TGF-β is involved in the effect, but it does not indicate which isoform is primarily responsible. The three isoforms
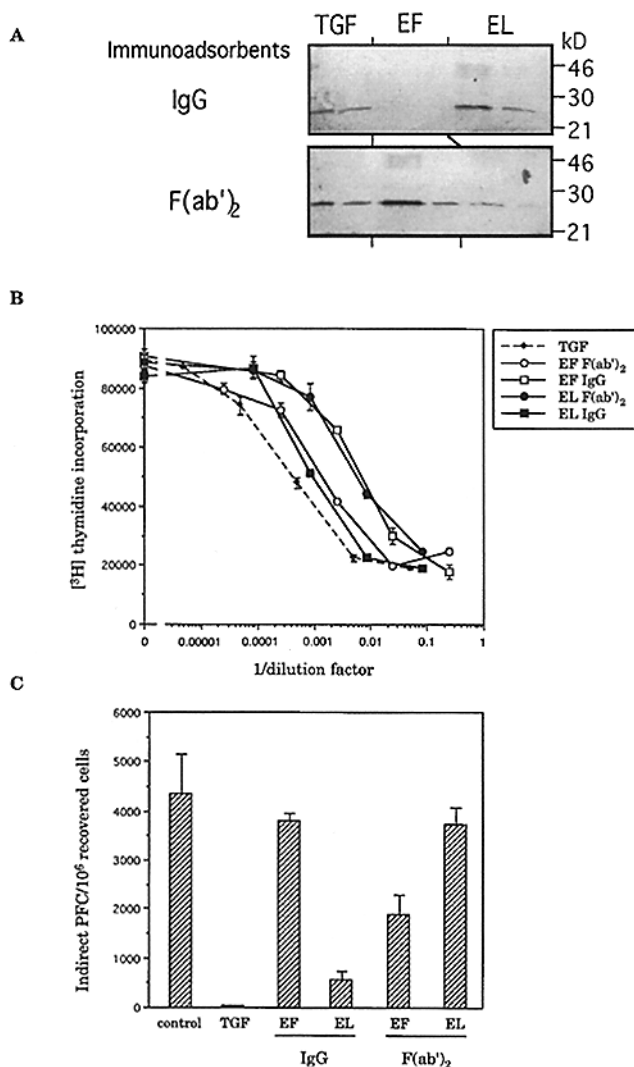
A

Immunoadsorbents

IgG

F(ab')₂

TGF EF EL kD
— 46
— 30
— 21
— 46
— 30
— 21

B

[³H] thymidine incorporation

1/dilution factor

— TGF
— EF F(ab')₂
— EF IgG
— EL F(ab')₂
— EL IgG

C

Indirect PFC/10⁶ recovered cells

control TGF EF EL EF EL

IgG   F(ab')₂

Figure 7. TGF-β binds to IgG, but not to F(ab')₂ fragments. Porcine TGF-β1 was incubated with IgG or F(ab')₂ immunoadsorbents. Effluents (*EF IgG, EF F(ab')₂*) and acid eluates (*EL IgG, EL F(ab')₂*) were recovered. Fractions (30 and 5 μl) and TGF-β1 (1 and 0.3 ng) were analyzed by Western blotting with chicken anti–porcine TGF-β1 (*A*), for biological activity on LPS-stimulated proliferative B cell response (*B*), and on the secondary in vitro anti-SRBC response (final dilution = 1:200) (*C*). Results are expressed as the number of indirect PFC per 10⁶ recovered cells (means ± SEM of duplicate cultures).

of TGF-β bind to the same receptors, although with variable affinities (35), and have similar biological activities. We did not therefore attempt to characterize the TGF-β isoforms present in the IgG-BF preparations. The fact that neutralizing antibodies reversed suppression suggested either that TGF-β–like molecules were present in the IgG-BF preparations, or that the production of TGF-β was induced by IgG-BF. Indeed, these antibodies neutralized endogenously produced mouse TGF-β since their addition to activated B cells or to SRBC-stimulated splenocyte cultures enhanced cell responses as already observed (40). To discriminate between the two possibilities, it was necessary to physically separate TGF-β from sFcγR in IgG-BF prepara-

tions. This was achieved on a gel filtration column. When the different fractions were tested for suppressive activity, the fractions containing the bulk of TGF-β were active on antibody production, in contrast to those containing sFcγR. Exogenous TGF-β present in IgG-BF preparations seems therefore responsible for the inhibition of B cell responses and antibody production. It is noteworthy that, after gel exclusion chromatography, the bulk of TGF-β and the suppressive activity were present in the high molecular weight fractions of the gel exclusion column (>150 kD). These results are compatible with the fact that TGF-β forms complexes at neutral pH, as observed by others (26).

Immunosuppressive factors that bind IgG and not F(ab')₂ have been described by several groups in supernatants of cells secreting sFcγR such as mouse T cells and T cell lines and human mononuclear cells or neutrophils (7–14). They were isolated using affinity chromatography with insolubilized IgG. The question thus arises whether the results obtained with IgG-BF isolated from cell lines secreting recombinant sFcγR can be generalized to the natural factors. In the present work, Western blotting analysis showed that 25-kD TGF-β was detectable in IgG-BF isolated from supernatants of mouse activated T cells. It is noteworthy that suppressive 19–23-kD components have been previously identified in similar IgG-BF preparations (41). Components migrating above the 25-kD band were detected by Western blotting in porcine TGF-β, as well as in T cell–produced IgG-BF and in IgG-BF purified from the culture media of IVC2 and 6/9CII cell lines (Fig. 1 *D*). Their identity is presently unknown. A cross-reactive 35-kD contaminant has been already described in human rTGF-β (42).

Finally, our data suggest that the presence of TGF-β in IgG-BF preparations is most probably caused by selective binding of TGF-β to IgG. Indeed, we demonstrated that purified porcine TGF-β1 is absorbed on IgG-Sepharose, and not on F(ab')₂-Sepharose, and recovered in the acid eluates of IgG-Sepharose. The murine TGF-β–like suppressive factor present in IgG-BF purified from culture medium of the Cu1B3 cell line also bound to IgG and not to F(ab')₂ fragments of IgG since suppressive activity was eluted from IgG-Sepharose (Fig. 1 *A*), and not F(ab')₂-Sepharose (data not shown), as already observed (19), and TGF-β was found in the acid eluates from IgG-Sepharose (Fig. 1 *C*). Stach and Rowley (26) recently demonstrated that the IgG-mediated suppression of in vitro cytotoxic T cell responses is caused by IgG-bound TGF-β. These authors reported that in immune serum, TGF-β circulates with IgG antibodies at a ratio of 2–10 ng TGF-β for 1 mg IgG. TGF-β–induced T cell suppression was inhibited by anti-TGF-β antibodies, but also by rabbit IgG and anti-FcγR antibodies, and it required interaction with macrophage FcγR (26). Our own data emphasize the role of TGF-β and its binding to IgG in the modulation of B cell responses.

TGF-β is synthesized by many cell types and secreted almost exclusively as a biologically inactive or "latent" complex (30, 43) that can be activated by exposure to acidic or alkaline environments, by heating at 100°C, by treatment

with chaotropic agents, and by the limited action of proteases and glycosidases (29, 44). TGF-β, recovered in the acid eluates of IgG-Sepharose, was suppressive for B cell proliferation and antibody production and its effect was inhibited by antibodies reacting with active TGF-β, suggesting that TGF-β was present in an active form. It is unclear whether it was already active in the starting culture medium. Many studies have shown that latent TGF-β must be acidified to <pH 4 to obtain efficient activation (44). In this study, all purification steps of recombinant sFcγR were performed >pH 4. The culture medium of Cu1B3 cell line grown on hollow fibers was suppressive (Fig. 1 A) and contained TGF-β, as demonstrated by Western blotting (Fig. 1 D). Similar results were obtained with the culture media of 6/9CII and IVC2 cell lines (data not shown). It is possible that the culture of transfected cells in bioreactors at high cell density for several weeks may lead to a certain degree of cell death and to the release of proteases, resulting in activation of latent TGF-β. The fact that the crude culture medium of the 6/9CII cell line grown in bioreactors inhib-

ited the proliferation of the mink epithelial CCL64 cell line and that its acidification did not increase its antiproliferative activity on CCL64 reinforce this possibility (data not shown).

The binding of TGF-β to complexed IgG may have major biological significance. The role of the TGF-β–binding proteins is diverse. For instance, latent TGF-β circulates in serum bound to α2-macroglobulin, which may protect it from activation (31), and sulphate proteoglycan decorin inhibits TGF-β activity (34). In contrast, association of murine TGF-β to α-fetoprotein results in an active suppressor factor (32), as does that of human TGF-β to fibronectin (33). Immune complexes of IgG antibodies may carry latent or activated TGF-β (26) and interact with FcγR in their membrane-associated or soluble forms (14, 18). TGF-β delivered by IgG to activated lymphocytes and macrophages may mediate important biological effects, both in vitro and in vivo (26). TGF-β therefore appears to be a major factor mediating the negative regulatory function exerted by IgG antibodies.

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