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Strong Mitogenic Effect for Murine B Lymphocytes of an Immunosuppressor Substance Released by *Streptococcus intermedius*

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A noncytotoxic protein substance, produced by *Streptococcus intermedius*, with very potent immunosuppressive properties (F3'EP-Si) was tested for lymphocyte mitogenic activity. Although devoid of T-cell mitogenicity, F3'EP-Si stimulated proliferation and led to high numbers of plaque-forming cells in cultures of normal or T-cell-depleted, small or large splenic B cells from both lipopolysaccharide-responding and -nonresponding mice. The B-cell mitogenic activity of F3'EP-Si was quantitatively comparable to that of lipopolysaccharide, and the simultaneous exposure to both mitogens stimulated additive B-cell responses. Injection of F3'EP-Si into normal mice resulted in increased numbers of spleen cells, higher rates of mitotic activity, and very large numbers of plaque-forming cells, predominantly of the immunoglobulin G2a and -b isotypes. In preliminary experiments, the analysis of surface markers among the lymphocytes participating in the blastogenic response in vivo revealed a T-cell component in the response to F3'EP-Si. These observations are discussed in the context of the immunosuppressive activity of this and other microbial substances.

We previously observed (3, 4) that a protein secreted by *Streptococcus intermedius* (F3'EP-Si) is a potent suppressive substance against the in vitro proliferative responses of human peripheral blood mononuclear cells and against the in vivo primary antibody response of C57BL/6 mice to sheep erythrocytes. Two observations led us to assume that the immunosuppression by F3'EP-Si was mediated by a pathway of overstimulation or excessive help and subsequent generation of T suppressor lymphocytes (3): (i) the suppressive effect of F3'EP-Si was more evident when the protein was tested under conditions favorable for reactivity and maturity of the target system, and (ii) the effects of F3'EP-Si in immune responses could be switched from suppressive to stimulatory when the time of exposure or target system or both were reduced, even under conditions of weak stimulation. This assumption was substantiated by the fact that F3'EP-Si-treated animals exhibited splenomegaly with histological patterns consistent with an adjuvantlike effect (5).

Because it had been demonstrated that other microorganisms, including *Brucella melitensis* (22), *Leishmania* spp. (21), *Trypanosoma cruzi* (13; P. Minóprio, H. Eisen, L. Forni, R. Imperio-Lima, M. Joskwicz, and A. Coutinho, submitted for publication), and lactic dehydrogenase virus (7), are B-cell mitogens, we decided to investigate whether F3'EP-Si was also a B-cell mitogen, which could explain the observed stimulatory immunosuppressive effects of this substance. We report here results demonstrating strong B-cell mitogenicity in vitro and in vivo which, in preliminary experiments, appeared to result in the secondary participation of T lymphocytes.

MATERIALS AND METHODS

Preparation of F3'EP-Si. F3'EP-Si was prepared as previously described (3–5). Briefly, *S. intermedius* was cultured anaerobically over a dialysis membrane in close contact with tryptone-glucose agar culture medium for 24 h at 37°C. The dialysis membranes were then washed with 0.05 M potassium phosphate buffer, and the wash was clarified by ultracentrifugation and concentrated by vacuum dialysis. F3'EP-Si was then obtained out of crude material by preparative isoelectrofocusing in a sucrose gradient, and its biological activity was evaluated by its ability to inhibit the primary immune response of C57BL/6 mice against sheep erythrocytes when injected into the animals 2 days before immunization. A unit of biological activity (UBA) was defined as the dose of F3'EP-Si that reduced by 50% the numbers of directly hemolytic splenic plaque-forming cells (PFCs) of treated animals. All preparations of the substance were sterilized by irradiation and diluted in balanced salt solution before use in tissue culture.

Mice. C57BL/6, C57BL/10SrCr, C3H/He, C3H/Tif, and A/J mice were bred at the Pasteur Institute.

Cell preparation and culture. Spleen cells were prepared in single suspension as previously described (1). T-cell removal was achieved by cytotoxicity with the monoclonal rat anti-mouse Thy-1 antibody J.1j (μ and κ chains) (kindly provided by J. Sprent, Scripps Clinic and Research Foundation, La Jolla, Calif.) and rabbit complement. T-cell enrichment was obtained by passage over nylon-wool columns (14), with cell yields varying from 15 to 20%. Isolation of small and large splenic lymphocytes was performed on discontinuous Percoll gradients as described previously (16, 17). An interleukin-2 (IL-2)-containing conditioned medium was obtained from the cell-free supernatants of rat spleen cells stimulated with 5 μg of concanavalin A (ConA) per ml for 24 h at 5×10^6 cells per ml. These supernatants were titrated for IL-2 content in a biological assay with IL-2-dependent, established T-cell lines.

Cells were cultured at 4×10^5 per ml, unless otherwise indicated, in RPMI 1640 medium supplemented with 10% fetal calf serum, 5×10^{-5} M 2-mercaptoethanol, and anti-

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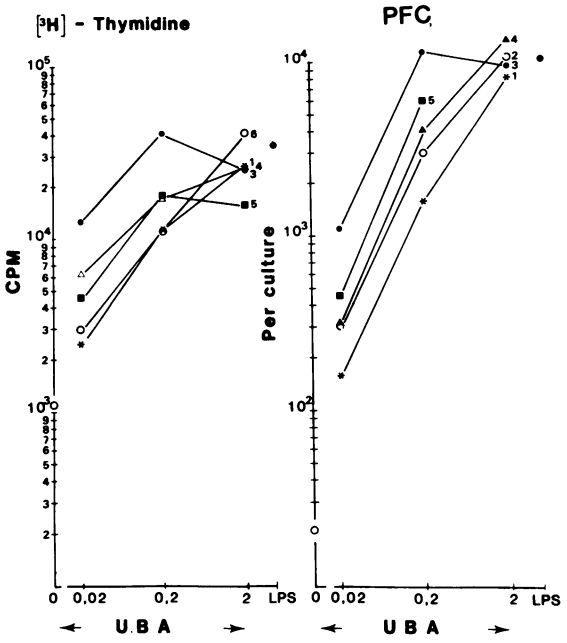

FIG. 1. Mitogenicity of F3'FP-Si to normal spleen cells. C57BL/6 spleen cells were cultured (8 × 10^4 cells per well) in the presence of the indicated concentrations (UBA) of five different preparations of F3'EP-Si. Parallel cultures were assayed for proliferation at 48 h and for polyclonal PFC responses at 72 h. The background values in unstimulated cultures, as well as the control responses to optimal concentrations (25 μg/ml) of LPS, are also indicated.

biotics, as described previously (1). The cultures were set up in 0.2-ml portions in the wells of microtiter plates and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

Mitogens. Lipopolysaccharide (LPS) from "*Streptococcus abortus* subsp. *equi*" was obtained from Difco Laboratories, Detroit, Mich., and ConA was obtained from Pharmacia, Uppsala, Sweden.

Assays. Proliferative responses were assayed by incorporation of tritiated thymidine (specific activity, 2 Ci/mmol; Amersham, England) after a 3-h pulse with 1 μCi per culture. Polyclonal B-lymphocyte responses were quantitated by the numbers of high-rate immunoglobulin-secreting cells detected as PFCs in the protein A assay (12). Rabbit antisera specific for each isotype of the mouse immunoglobulins used as developing antibodies in the plaque assay have been characterized (8). In each experiment, all assays were done in triplicate cultures, and the results are expressed as mean values. For simplicity, the standard errors of the means are not shown because in every case they were less than 15% of the mean.

Dual-parameter analysis of cell size and surface markers was performed by flow cytofluorometry with an FACS Analyzer I (Becton Dickinson and Co., Paramus, N.J.) and the following monoclonal antibodies: rat anti-mouse κ chains [R33-18; immunoglobulin G (IgG)(κ) (R. Grützmann, Ph.D. thesis, University of Cologne, Cologne, Federal Republic of Germany, 1981)], rat anti-Lyt-2 [53-6.7; IgG(κ) (15)], and rat anti-L3T4 [H-129.19.69; IgG(κ) (19)]. These were biotinylated as described previously (10), and surface stainings with avidin-fluorescein were performed as previously described (10).

RESULTS

In vitro experiments. (i) F3'EP-Si is strongly mitogenic for murine splenocytes. As indicated above, we had several reasons to suspect that F3'EP-Si would have stimulatory effects on lymphocytes. In an initial series of experiments, various preparations of F3'EP-Si with previously quantitated and somewhat different biological activity in terms of suppression of in vivo PFC responses to sheep erythrocytes were titrated in cultures of normal murine spleen cells in parallel with optimal doses of a known B-cell mitogen, LPS. All five preparations induced marked proliferation of spleen cells, at levels comparable to those observed in response to LPS (Fig. 1). Peak responses represented a 40-fold stimulation of thymidine uptake over background values. A first indication of the cell type selectivity of the mitogenic activity of F3'EP-Si was obtained by recording the number of activated effector B cells (PFCs) in these cultures. The five different preparations all induced a very large number of PFCs in spleen cell cultures, again at levels comparable to LPS-induced responses and nearly 1,000-fold higher than those recorded for unstimulated cultures (background values) (Fig. 1).

(ii) F3'EP-Si is a murine B-cell mitogen. To evaluate whether the lymphocyte mitogenic activity described above, which appeared to include B lymphocytes, was dependent on or encompassed T cells as well, a purified preparation of F3'EP-Si was titrated on T-cell-depleted spleen cell cultures in parallel with cultures of untreated cells. There was little or no difference in the proliferative and PFC responses of

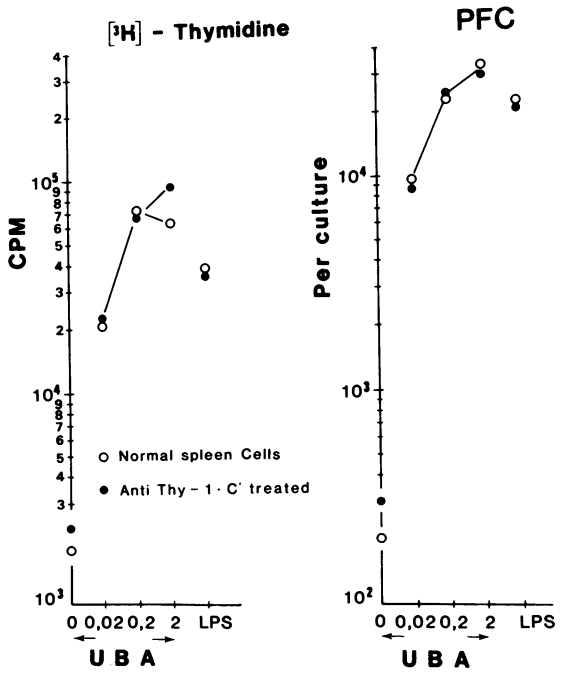

FIG. 2. T-cell-independent mitogenic activity of F3'EP-Si. Spleen cells from C57BL/6 mice were either treated with anti-Thy-1 and complement (●) (50% viable cell recovery) or left untreated (○) and were then cultured (8 × 10^4 cells per well) with the indicated concentrations (UBA) of F3'EP-Si. Proliferative responses were measured at 48 h, and the polyclonal PFC responses were assayed at 72 h. The control responses to LPS are also shown. The proliferative responses to ConA were 37,900 and 1,900 cpm per culture in untreated and T-cell-depleted cultures, respectively.

splenic B cells regardless of the presence of T lymphocytes (Fig. 2). The same was true for the control responses to LPS, whereas T-cell-depleted cultures failed to develop proliferative responses to the T-lymphocyte mitogen ConA (data not shown).

Independent confirmation for the lack of direct effects of F3'EP-Si on normal T cells was obtained in experiments in which T-cell-enriched preparations were cultured with a wide range of concentrations of the substance in the presence or absence of IL-2-containing conditioned medium (Table 1). We conclude, therefore, that F3'EP-Si is a strong B-lymphocyte mitogen and appears devoid of direct mitogenic or otherwise stimulatory effects on T lymphocytes. In an attempt to further characterize the target B cells in terms of the mitogenic activity of F3'EP-Si, we analyzed the responses of small and large spleen cells, since B-cell mitogens with activity limited to large, previously induced cells have been described (2). However, F3'EP-Si showed comparable activating properties for both large and small B lymphocytes from a normal murine spleen (Fig. 3).

(iii) F3'EP-Si is a B-lymphocyte mitogen for a cell population different from those responding to LPS. Because the activity of F3'EP-Si is qualitatively and quantitatively very similar to that of LPS, putative contaminations with this well-characterized mitogen should be controlled. C57BL/10ScCr mice carry a defective allele at the *lps* locus in chromosome 4 (9) which results in a complete lack of reactivity to this mitogen and invariably diminished responses to other B-cell activators. However, C57BL/10ScCr B cells proliferate and differentiate to PFCs if stimulated by F3'EP-Si at levels which are comparable to those observed in C57BL/6 mice (Fig. 4). In contrast, LPS-induced responses are excellent in C57BL/6 mice and completely absent in C57BL/10ScCr spleen cells. We conclude that the B-cell mitogenic activity of F3'EP-Si is not due to contamination by LPS-like substances.

An alternative evaluation of the contribution of LPS to the polyclonal responses to F3'EP-Si was provided by mixing experiments. Titrating LPS to find the concentrations inducing optimal proliferation and adding various F3'EP-Si preparations to near optimal concentrations of LPS allowed us to determine whether the mitogenic activity of the latter is effected via the same or similar mitogenic principles. Thus, assuming that all LPS-reactive lymphocytes were already responding to optimal LPS concentrations, the finding of additive effects in the response to mixtures of LPS and F3'EP-Si would provide strong indications for additional

TABLE 1. Absence of T-cell mitogenicity of F3'EP-Si detectable in vitro[a]

Culture medium	[^{3}H]thymidine uptake (cpm/culture) with:					
	No additions	F3'EP-Si (UBA)				ConA
		0.002	0.02	0.2	2	
Without IL-2	288	295	186	1,446	4,327	47,304
With IL-2	922	996	835	1,047	1,444	55,380

[a] Spleen cells from C3H/He mice were enriched for T lymphocytes by passage over nylon-wool columns and cultured (6×10^4 cells per ml) with either 5 μg of ConA per ml or the listed concentrations of F3'EP-Si in the presence or absence of IL-2-containing conditioned medium, as indicated. Proliferative responses were measured on days 2, 5, and 6 of culture. Only day 2 results are shown since no responses to F3'EP-Si were detected on either day 5 or day 6. The low level of proliferation observed with the highest concentrations of F3'EP-Si was most likely due to the response of contaminating B cells, as supported by PFC responses detected on day 6 and by the inhibition of proliferation upon the addition of IL-2-containing medium.

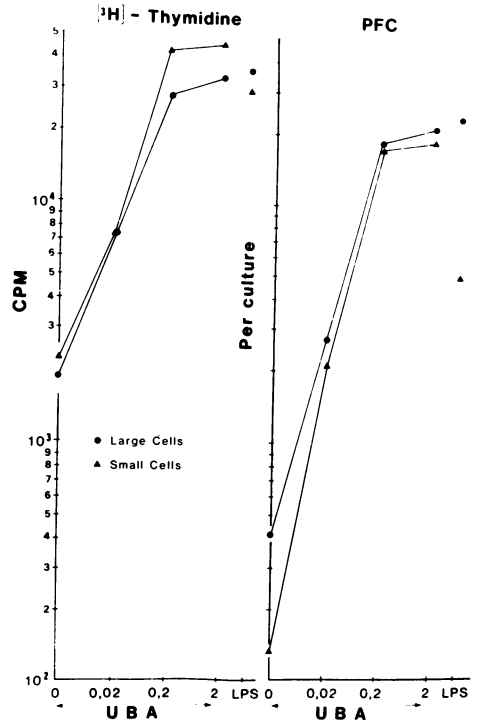

FIG. 3. Stimulation of both large and small B cells by F3'EP-Si. Spleen cells from C57BL/6 mice were separated in Percoll gradients, and the fractions with $\rho \geq 1.08$ (small) and $1.07 \geq \rho \geq 1.06$ (large) were collected and cultured (6×10^4 cells per well) with the indicated concentrations (UBA) of F3'EP-Si or with LPS as a control. The assays were done as described in the legend to Fig. 2.

lymphocytes being activated by mitogenic substances other than LPS. This was in fact the case for five independently prepared batches of F3'EP-Si tested by this protocol (Fig. 5). In every case, clear additive effects were observed which, in addition to demonstrating the nondependence of F3'EP-Si mitogenicity on LPS, suggest, very interestingly, that this particular substance stimulates B-lymphocyte populations that are largely different from those responding to LPS.

In vivo experiments: mitogenicity of F3'EP-Si blastogenesis of B and T cells and restricted isotype pattern of PFCs. The mitogenic effects of F3'EP-Si detected in vitro would have greater significance for the interpretation of the suppressive effects previously described (3–5) if they were observed in vivo as well.

Normal C3H mice were injected with F3'EP-Si, and 4 days later, various parameters of lymphoid activity in their spleens were analyzed in parallel with activity in control mice treated with saline. Injection of F3'EP-Si resulted in hypercellularity and in increased in vivo mitotic activity in the spleens (Table 2). Most importantly, a considerable increase in the numbers of total PFCs was detected in the spleens, demonstrating the B-cell mitogenicity of F3'EP-Si in vivo. It is interesting to note the isotype specificity of this polyclonal B-cell response in vivo. It has been shown (6) that injection of LPS results in a similar response to that described here for F3'EP-Si, except for the isotype pattern of the polyclonal PFCs. Whereas LPS preferentially induces IgG3 and IgG2b among all IgG subclasses, F3'EP-Si primarily activated IgG2b and IgG2a, in a manner that has been found only in the polyclonal B-lymphocyte responses after injection with *T. cruzi* (13).

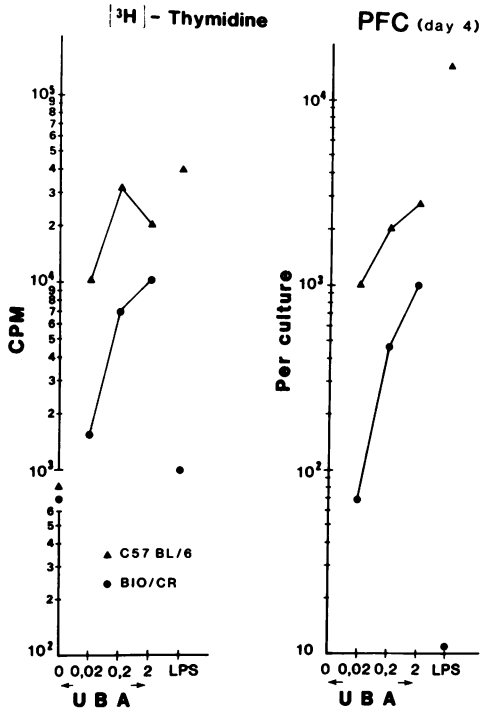

FIG. 4. Mitogenicity of F3'EP-Si is not due to LPS contamination. Spleen cells from either C57BL/6 (LPS responding) (▲) or C57BL/10ScCr (LPS nonresponding) (●) mice were cultured (8 × 10^4 cells per well) with the indicated concentrations (UBA) of F3'EP-Si or with optimal LPS concentrations as a control. Proliferative and polyclonal PFC responses were assayed on days 2 and 4, respectively.

Because in the latter case this peculiar isotype pattern was attributed to T-cell participation in the response, we investigated this possibility by studying the state of activation of T lymphocytes in the spleens of F3'EP-Si-injected mice. This was done by flow cytofluorometry by using cell volume as a parameter of blastogenic activity and performing dual-parameter analysis for T-cell-specific surface markers (Fig. 6 and Table 3). Injection of F3'EP-Si resulted 4 days later in a considerable increase in the total number of spleen cells and, more importantly, in the frequency of activated, large cells. The analysis of each lymphocyte population distinguished by surface markers confirmed that a blastogenic response of B cells took place and, in addition, revealed a notorious T-lymphocyte response in both the L3T4 (helper) and Lyt-2 (suppressor) compartments. The latter was clearly more stimulated than was the helper-inducer subpopulation.

These results established that F3'EP-Si stimulates in vivo a considerable number of B and T cells to blast transformation and increases mitotic activity under the same conditions as those which result in strong immunosuppression.

DISCUSSION

The present results establish the B-cell mitogenic properties of F3'EP-Si, a noncytotoxic protein substance, produced by *S. intermedius*, with very potent immunosuppressive properties, that acts in conjunction with the effects of the mitogenic or antigenic stimulus and the reactivity of the target immune system (1). Immunosuppressive effects have been demonstrated in the in vitro responses of human lymphocytes and in the in vivo primary antibody response of mice to a thymus-dependent antigen (3). More recently, we also observed very significant prolongations of skin graft survival in murine allogenic combinations as a result of systemic administration of F3'EP-Si (M. Arala Chaves, unpublished observations). As explicated above, some characteristics of F3'EP-Si action suggested the possibility that the suppression of the immune response induced subsequent to its administration could come about by a mechanism of excessive stimulation of the participating lymphocytes and subsequent generation of suppressor T lymphocytes (5).

As shown here by the study of purified lymphocyte populations, F3'EP-Si is a B-cell mitogen, but it displays no detectable, direct stimulatory properties for T lymphocytes in vitro. This might explain why we have not observed a macrophage-dependent immunosuppressor effect of F3'EP-Si (3), an effect that would be expected, unlike B-cell mitogenicity, if F3'EP-Si was primarily affecting T lymphocytes. Furthermore, F3'EP-Si was a competent mitogen for B cells from LPS-nonresponding mice, which excludes the possibility that its functional properties were due to endotoxin contamination in our preparations. Interestingly, B-cell responses to the simultaneous stimulation with F3'EP-Si and LPS stimulated largely distinct B-lymphocyte subpopulations. Similar observations have been reported for LPS, purified protein derivative, and dextran sulfate and interpreted as an indication of selective mitogen reactivity of B cells according to their differentiative stages (11).

It is important to emphasize that the mitogenic activity of F3'EP-Si was readily demonstrable in vivo under the same conditions as those which result in immunosuppression of immune responses. This lends further support to the hypothesis that these two immunological consequences of F3'EP-Si injection are interrelated.

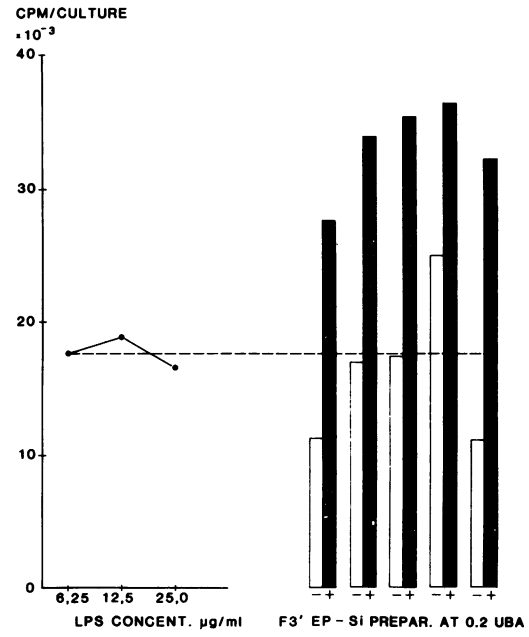

FIG. 5. Additive mitogenic effects of LPS and F3'EP-Si for murine spleen cells. C57BL/6 spleen cells were stimulated in culture (8 × 10^4 cells per well) with the indicated concentrations of LPS (●), with 0.2 UBA of each of five different preparations of F3'EP-Si per ml (□), or with a mixture of 6.25 µg of LPS per ml and 0.2 UBA of each of the F3'EP-Si preparations per ml (■). Proliferative responses were measured on day 2 of culture.

TABLE 2. B-cell mitogenic activity of F3'EP-Si in vivo[a]

Treatment	No. of cells/spleen	Proliferative activity (cpm/2 × 10^5 cells)[b]	No. of isotype-specific PFCs/spleen (10^3)[c]						
			Total	IgM	IgG3	IgG1	IgG2b	IgG2a	IgA
Untreated	115 × 10^6	19,625	378	243.2	20.4	8.1	19.5	16.8	69.6
F3'EP-Si	165 × 10^6	51,050	1,766.6 (4.7)	940 (3.9)	137.5 (6.7)	60.5 (7.1)	250 (12.8)	314.8 (18.7)	63.8 (0.92)

[a] C3H/He mice were injected intraperitoneally with 8 UBA of F3'EP-Si or left untreated. Four days later, spleen cell suspensions were individually assayed for proliferative activity and numbers of PFCs for all isotypes. The results are the mean values for two injected animals. For technical reasons, only one control animal was assayed, but the levels of activity in this mouse were not significantly different from those in a very large number of animals of the same strain and age assayed over the last 3 years.

[b] Freshly prepared spleen cells were directly pulsed in culture (10^6 cells per ml) with 1 μCi of tritiated thymidine for 3 h.

[c] Isotype-specific PFCs were determined in the protein A plaque assay. The numbers in parentheses represent the ratios of the numbers of PFCs in infected mice to those of the same isotypes in the controls.

On the other hand, although B-cell mitogenicity could explain the suppression of humoral immune responses, as is the case with LPS if it is administered 2 days before the specific antigen (18), it was difficult to accommodate in the same framework the suppression of T-cell-mediated responses in the absence of demonstrable T-cell mitogenicity. In this context, we decided to investigate the levels of T-cell activity in vivo after injection of F3'EP-Si.

The results very clearly demonstrated a blastogenic response of splenic T cells after in vivo administration of F3'EP-Si. In light of the negative in vitro results, we interpret this in vivo T-cell response as being a secondary consequence of the B-cell mitogenic response. We recently showed that activated B cells are excellent stimulators of autologous T lymphocytes of both the L3T4- and Lyt-2-positive classes (M. Björklund, A. Beretta, A. Coutinho, and M. Gullberg, Eur. J. Immunol., in press). Furthermore, we have observed that such T cells, activated by syngeneic B-cell blasts, display effector functions, particularly of the suppressor and cytotoxic type (Björklund et al., in press). As shown here, injection of F3'EP-Si also preferentially activates Lyt-2-positive cells, and it is very likely that such autoreactive suppressor lymphocytes are very efficient in limiting clonal expansion induced by any antigen administered at this time point. Future experiments are needed to establish whether (i) this in vivo T-cell response is indeed an autologous reaction to activated B cells (ii), the activated Lyt-2-positive cells are in fact suppressor-cytotoxic effector cells, and (iii) such Lyt-2-positive cells are the mediators of suppressive activities in the various test systems previously used (primary PFC responses to sheep erythrocytes and allogenic skin graft rejection). It is interesting, however, that a very similar situation has been reported for mice infected with *T. cruzi* (13; Minóprio et al., submitted) and with lactic dehydrogenase virus (7). A large blastogenic B-cell response is followed by a stage of extensive T-lymphocyte activation

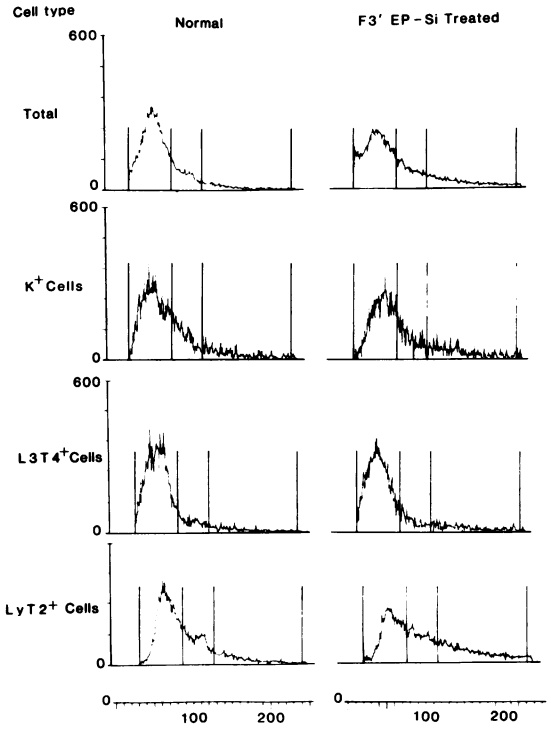

FIG. 6. In vivo mitogenic response of B and T cells to injection of F3'EP-Si. C3H/Tif mice were injected intraperitoneally with 1.6 UBA of F3'EP-Si or left untreated. Four days later, spleen cells were recovered and analyzed for volume in a FACS Analyzer I. Volume distributions were also analyzed in cell populations "gated" for each of the three markers indicated (membrane K chains or L3T4 or Lyt-2 antigens) after surface staining with the corresponding monoclonal antibodies coupled with biotin followed by staining with avidin-fluorescein. The results shown are for a pool of cells from two injected mice and two untreated controls.

TABLE 3. In vivo blastogenic response of splenic T and B cells to F3'EP-Si

Surface marker and treatment[a]	No. of cells/spleen (10^6)	Volume distribution (% of total)[b]			No. of activated cells/spleen (10^6)[c]
		Small	Medium	Large	
Total nucleated cells					
Untreated	88.7	72.3	18.9	8.8	24.5
F3'EP-Si	146.2	61.1	20.9	17.7	56.4
K chains					
Untreated	42.2	58.7	26.4	14.7	17.3
F3'EP-Si	64.9	42.2	27.4	29.6	37.0
L3T4					
Untreated	22.4	80.7	12.0	7.2	4.3
F3'EP-Si	24.0	75.4	12.2	12.0	5.8
Lyt-2					
Untreated	9.3	64.7	22.9	12.2	3.2
F3'EP-Si	11.3	56.8	23.3	19.4	4.8

[a] As described in the text; the percentages of positive cells determined with the FACS Analyzer for untreated and F3'EP-Si-injected mice, respectively, were 47.6 and 44.4 for K-positive cells, 25.2 and 16.4 for L3T4-positive cells, and 10.5 and 7.7 for Lyt-2-positive cells. Treatments were pools of spleen cells from two mice injected 4 days earlier with 8 UBA of F3'EP-Si and from two age-matched controls.

[b] Percentage of total cells bearing the surface marker indicated; the three volume classes were defined as shown in Fig. 6.

[c] Considering all medium and large cells as activated.

(Minóprio et al., submitted) simultaneous with the establishment of immunosuppression (20). Reinforcing this parallel, it is interesting that the predominant classes of immunoglobulin in the polyclonal B-cell response to *T. cruzi* infection are the IgG2a and IgG2b isotypes (13), which are relatively T-cell dependent (8) and precisely the dominant isotypes which occur in response to F3'EP-Si, as shown here.

Underlying all of these questions, there is a most interesting possibility that different microorganisms can induce the generation of T suppressor cells and thus immunosuppression through polyclonal B-cell activation; it was recently described for leishmaniasis (21) that B-cell activation can induce T-cell suppression.

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