COMPLEMENT-DEPENDENT B-CELL ACTIVATION BY COBRA VENOM FACTOR AND OTHER MITOGENS?*

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Bone marrow-derived cells (B cells),[1] i.e. the precursors of antibody-secreting cells, are distinguished by a dense coat of surface immunoglobulin able to bind antigen specifically (1–4). In addition, an important subpopulation of B cells (complement-receptor lymphocytes [CRL]) carries membrane receptors for activated C3 capable of binding any antigen-antibody complex which functions as vehicle for C3b (5–8).

It was recently proposed that two distinct signals are required for triggering B lymphocytes along the pathway of antibody formation: (*a*) the specific binding of antigen to the immunoglobulin receptors and (*b*) the interaction of activated C3 with the complement receptors (9). In the context of this hypothesis it was postulated that release of proteases from stimulated thymus-derived lymphocytes (T cells) would generate active split products of C3 which could in turn bind to B lymphocytes, thus rendering them susceptible to activation by T-dependent antigens. The direct activation of B cells by T-independent antigens (capable of eliciting an antibody response in the absence of thymus-derived cells) was thought to be linked to the presumed capacity of such molecules to activate C3 via the bypass mechanism. In fact, such a correlation seems to hold true for a number of T-independent antigens and B-cell mitogens.[2]

If B-cell activation depends on such a mechanism, one would predict not only that T-independent antigens should be bypass activators, but also that bypass activators

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[1] *Abbreviations used in this paper*: B, bone marrow-derived (cells); Con A, concanavalin A; CRL, complement receptor lymphocytes; crT, cortisone-resistant thymus cells; EAC, erythrocyte-antibody-complement complexes; FBS, fetal bovine serum; hiFBS, heat-inactivated FBS; zaFBS, zymosan-absorbed FBS; GP, guinea pig; LNC, lymph node cells; LPS, lipopolysaccharide W *E. coli* 0111:B4; M, macrophages; 2-ME, 2-mercaptoethanol; PFC, plaque-forming cells; PHA, phytohemagglutinin P; PWM, pokeweed mitogen; SC, spleen cells; SEPH-VF, sepharose-coupled VF; SEPH-VFSF, serum factor-converted SEPH-VF; SRBC, sheep red blood cells; T, thymus-derived (cells); [^{3}H]T,[6-^{3}H]thymidine; VF, cobra venom factor.

[2] Bitter-Suermann, D., U. Hadding, M. Dierich, U. Schorlemmer, U. Limbert, and P. Dukor. Manuscript submitted for publication.

might be effective B-cell mitogens and could substitute for the T-cell requirements of unrelated antigens, provided the bypass-inducing substance could bind in a suitable way to B-cell membrane components. Moreover, it would be expected that B-cell activation in vitro might depend on the availability of exogenous complement.

In the work reported here we used purified cobra venom factor (VF, a well defined and potent bypass inducer [10–12]) to test this hypothesis.

Materials and Methods

Mitogens.—VF was purified chromatographically from lyophilized cobra venom (Naja Naja) (Miami Serpentarium Labs, Miami, Fla.) by previously detailed methods (12). The final preparation contained approximately 3 μg protein per unit of activity (11). In order to exclude a contamination with lipopolysaccharides, the purified VF preparation was tested for pyrogenicity in rabbits using standard pharmaceutical procedures.[3] Coupling of VF to Sepharose 4B (Pharmacia, Uppsala, Sweden) was carried out as described before (13). SEPH-VF was suspended in an equal volume of veronal buffered saline (0.15 M, pH 7.4) to give a concentration of 0.6 mg bound VF/ml. Phytohemagglutinin P (PHA, Difco Laboratories, Detroit, Mich.), Concanavalin A (Con A, Calbiochem, San Diego, Calif.), pokeweed mitogen (PWM, Grand Island Biological Corp., Grand Island, N. Y.), and lipopolysaccharide W *E. coli* 0111:B4 (LPS, Difco) were obtained commercially. Except for SEPH-VF, 10 μl of mitogen dissolved in complete culture medium was added to the lymphocyte cultures at the beginning of the incubation period.

Animals.—Specific pathogen-free mice of the lines nu/nu (congenitally athymic "nude," fourth backcross generation with Balb/c), nu/+ = (nu/nu × Balb/c) F_1, and C3CF$_1$ = (C3H × Balb/c) F_1 were purchased from Bomholtgård, Ry, Denmark and maintained in a barrier-sustained isolation unit. C5-deficient B10.D2-old/Sn mice and Pirbright guinea pigs (GP) were obtained from Jackson Laboratory, Bar Harbor, Maine, and Tierfarm AG, Sisseln, Switzerland, respectively. Within each experiment test and control groups were matched for sex and age.

Cell Suspensions.—Single cell suspensions for tissue culture experiments were prepared using standard aseptic techniques (14). Cortisone-resistant thymus cells (crT) were obtained from 6- to 8-wk old nu/+ donors which had been injected intraperitoneally with 125 mg/kg cortisone acetate 48 h before (15). Lymph node cells (LNC) were prepared from pooled mesenteric, axillary, and inguinal nodes of 8- to 12-wk old nu/+ and nu/nu mice. Nu/nu LNC were filtered through a glass wool column in order to remove nonviable cells (14). Nu/+ and nu/nu LNC suspensions were contaminated with less than 1% mononuclear phagocytic cells, whereas spleen cell suspensions (SC) from 8- to 12-wk old nu/+ and nu/nu donors were found to contain 3.0 ± 0.9 and 7.4 ± 2.4% macrophages (M), respectively (16). M were purified from gelatine-induced peritoneal exudates of 6- to 8-wk old nu/+ donors by adherence to plastic dishes (16). Thus, the different cell suspensions provided sources of T and B cells, and of M as follows: crT = T, nu/nu LNC = B, nu/+ LNC = T + B, nu/nu SC = B + M, nu/+ SC = T + B + M, nu/+ adherent peritoneal cells = M (16). For rosette assays, SC from 10- to 12-wk old C3CF$_1$ mice and mesenteric GP LNC from 7- to 8-wk old GP were prepared as described elsewhere (5).

Lymphocyte Cultures.—In order to assess mitogen-induced lymphocyte proliferation, triplicate cultures of nu/+ crT and of nu/+ or nu/nu LNC were set up in disposable 12 × 75-mm tubes (Falcon Plastics, Division of BioQuest, Oxnard, Calif.) containing each 1×10^6

[3] Dietrich, F. M., R. H. Gisler, C. Pericin, and G. Schumann. *Proc. Soc. Exp. Biol. Med.* In press.

cells in 1-ml culture medium (Eagle's MEM for suspension cultures, Flow, Irvine, Scotland) modified as indicated by Mishell and Dutton (17) and supplemented with 8% fetal bovine serum (FBS) (Rehatuin, Reheis Chemical Co., Chicago, Ill). The tubes were kept at 37°C in a CO_2 incubator (8% CO_2 in air). Every 24 h, 60 μl of a nutritive cocktail (14) was added. 16 h before harvest [6-^{3}H]thymidine ([^{3}H]T, 0.5 μCi/ml, sp act 23.3 Ci/mmol, Radiochemical Centre, Amersham, England) was added. TCA-precipitable radioactivity was measured using a liquid scintillation spectrometer. Results are expressed as counts per minute per tube. Details of the culture technique have been published before (14).

Induction of antibody formation to SRBC in vitro was studied using the conditions indicated by Mishell and Dutton (17). Triplicate cultures of 1.5×10^7 nu/+ or nu/nu SC were set up in 35 × 10 mm disposable Petri dishes (Falcon Plastics) containing 1 ml of the same medium as used for the tube cultures, but supplemented with 10% FBS. Composite cultures (16) consisted of 3×10^6 nu/+ crT and 7×10^6 nu/nu LNC which were added to approximately 3×10^5 adherent nu/+ peritoneal cells already attached to the dish. In some experiments, M were omitted and the LNC cultured in the presence of 50 μM 2-mercapto-ethanol (2-ME) (18). From the beginning, cultures were incubated with 3×10^6 SRBC and assayed for 19S plaque-forming cells (PFC) 3–5 days later using the microslide method of Berglund (19). In the case of cultures containing sepharose, it was easily possible to distinguish between "true" hemolytic plaques produced by PFC and "false" plaques due to dislocation of SRBC in the agar gel by sepharose particles. True plaques were readily identified microscopically by the presence of nucleated cells in the center of a hemolytic area, whereas false plaques were much smaller and completely translucent with sharply defined contours. Moreover, if SEPH-VF was plated with SRBC in the absence of cultured cells, no true hemolytic plaques could be observed.

Rosette Inhibition.—In order to study the possible interaction of activated C3 with CRL, SEPH-VF was converted with fresh mouse or GP serum to a SEPH-VFSF enzyme complex capable of cleaving C3 in the absence of divalent cations (12): SEPH-VF was washed three times (centrifugation 2,800 *g*, 10 min, 4°C) in excess medium RPMI 1640 (Grand Island). 0.5-ml packed SEPH-VF were then incubated with 6 ml fresh serum at 37°C for 60 min in an immersion rotator, and washed again four times in excess RPMI 1640. 5×10^6 $C3CF_1$ SC in 0.3 ml RPMI 1640 buffered with 28 mM HEPES (Calbiochem) and adjusted to pH 7.6 were mixed with 0.2 ml fresh mouse plasma (collected in EDTA adjusted with NaOH to pH 7.6, final concentration 10 mM) and 0.1 ml packed SEPH-VFSF (mouse) in the presence of 10 mM EDTA. Similarly, 10^7 GP LNC (filtered through glass wool in order to remove nonviable cells) were allowed to interact with fresh GP serum and SEPH-VFSF (GP) in the presence of EDTA. The tubes were incubated at 37°C for 30 min by use of the immersion rotator. The cells were then washed three times in medium RPMI 1640 with HEPES, resuspended in the same medium, and the viability determined by a trypan blue exclusion test. Suspensions containing 90–95% viable cells were adjusted to a cell density of 10^6/ml and allowed to form rosettes with SRBC which had been sensitized with rabbit amboceptor and C5-deficient serum from B 10. D2-old/Sn mice (EAC 1423). Except for the use of C5-deficient serum (dilution 1:2 or 1:4), the preparations of EAC and the rosette assay for detection of CRL were carried out exactly as described by Bianco et al. (5).

In another series of experiments, freshly prepared SC and LNC suspensions from 6- to 8-wk old $C3CF_1$ or nu/nu mice were preincubated with native, heat-inactivated or zymosan-absorbed homologous serum, or with serum from VF-treated mice (see below). 10^7 LNC in 0.3 ml medium RPMI 1640 were mixed with 0.2 ml serum in the presence or absence of 10 mM EDTA. After incubation at 37°C for 30 min in a waterbath, cells were washed three times in medium RPMI 1640 with HEPES and tested either for their rosette-forming capacity or for their proliferative potential in tissue culture.

Serum.—Native FBS, heat-inactivated (56°C, 60 min) FBS (hiFBS) and zymosan-absorbed

FBS (zaFBS) were all derived from the same frozen lot (see above). For absorption, equal volumes of FBS and boiled and washed zymosan (Nutritional Biochemicals Corp., Cleveland, Ohio) were incubated at 37°C for 1 h with an immersion rotator. After centrifugation at 4°C (3000 *g*, 30 min), absorbed FBS was stored at −80°C. The C3 content of the different FBS batches was estimated by testing them with GP complement components in an immune hemolysis assay (20). Native FBS contained 3.7×10^7 site-forming units C3 per ml. This value was taken as 100%. After heat inactivation of the serum, 70% of the C3 was still available, while after absorption with zymosan only 8.3% C3 remained.

Freshly collected mouse serum was heat inactivated or absorbed using the same conditions.

In Vivo C3 Depletion.—In order to obtain C3-depleted mouse serum (21), 8- to 12-wk old $C3CF_1$ mice received four intraperitoneal injections of 0.2 mg/kg VF at 8-h intervals. Thus, the total dose administered to each animal over a 24-h period corresponded to approximately 250 U of activity/kg. In some experiments control animals were treated with heat-inactivated (65°C, 30 min) VF. All mice were bled 48 h afrer the last injection.

RESULTS

In a first series of experiments, B-cell activation by VF was studied using a variety of culture systems. Since LPS preparations are known to be powerful stimulants of B-cell functions, possible contamination of VF with LPS had to be excluded first. For that purpose, the relative pyrogenicity of graded doses of VF and LPS was compared in rabbits. Whereas as little as 0.1 μg/kg LPS intravenous produced a significant fever response, VF was not pyrogenic at the highest dose level tested (10 μg/kg). The VF preparation used was thus not measurably contaminated with LPS. In lymphocyte cultures, VF was mitogenic for nu/+ LNC (T and B cells) and for nu/+ crT (T cells), as well as for nu/nu LNC (B cells). Peak incorporation of [^{3}H]T occurred on the 3rd day (Fig. 1). Maximum responses were elicited at a concentration of 16 μg VF/ml culture fluid (Fig. 2). Similarly, SEPH-VF was found to be equally mitogenic for both T and B cells.

In view of the marked B-cell activating properties of the two preparations, we sought to learn whether they could substitute for the role of T cells in antibody formation to a predominantly T-dependent antigen. Indeed, in T-cell deprived cultures, VF and SEPH-VF were able to restore responsiveness to SRBC. Thus, soluble VF greatly increased the number of PFC in SC cultures from athymic donors (Fig. 3). SEPH-VF was even more effective. In order to minimize possible phagocytosis of SEPH-VF by M, almost pure B cells (nu/nu LNC) were cultured in the presence of 2-ME, which is known to substitute operationally for M (18). If SEPH-VF was added (together with SRBC) to such cells, plaque responses were observed, which were similar to or even greater than those obtained in composite cultures containing optimal numbers of T and B cells and 2-ME (Fig. 4). On the other hand, both VF and SEPH-VF had an only moderate effect on the number of background PFC produced in cultures from which SRBC were omitted. Moreover, PFC production in T-cell sufficient SC cultures was not enhanced by the addition of VF. Some of these experiments are shown in Fig. 5. The results indicate, that the effect of SEPH-VF was

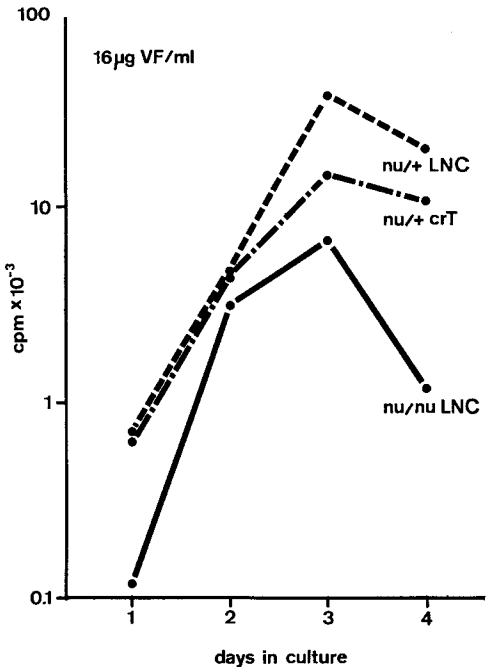

FIG. 1. Time course of [^{3}H]T incorporation into lymphocytes stimulated with 16 μg VF/ml culture fluid (cpm per culture). Symbols represent arithmetic means of triplicate cultures.

antigen-related, and that, at least in the model system employed, the VF preparations had functionally replaced the T cells.

If B-cell activation by VF and SEPH-VF were indeed linked to their presumed capacity of conferring activated C3 onto the C3 receptor of CRL, it might be possible to demonstrate blocking of the complement receptor after interaction with the bypass-activating substance in the presence of C3. This question was approached as follows: the presence of functional complement receptors on B cells can be readily visualized by cluster formation of CRL with EAC (5). As will be shown below, the rosette-forming capacity of freshly prepared lymphocyte suspensions is inhibited by preincubation with C3-sufficient homologous serum, unless EDTA is added to the incubate. Since C3 activation by VF depends on the presence of Mg^{++}, SEPH-VF had first to be converted with serum to give an enzyme capable of cleaving C3 in the absence of divalent cations (SEPH-VFSF, 12). Using this preparation, we studied the serum-dependent interaction of VF with CRL. GP LNC were preincubated in the presence of EDTA with homologous serum and with SEPH-VFSF (GP). As is demonstrated in Table I, the ability of such cells to form rosettes upon subsequent exposure to EAC 1423 was found to be greatly reduced. Similarly, a significant though less marked inhibition of rosette formation was obtained when

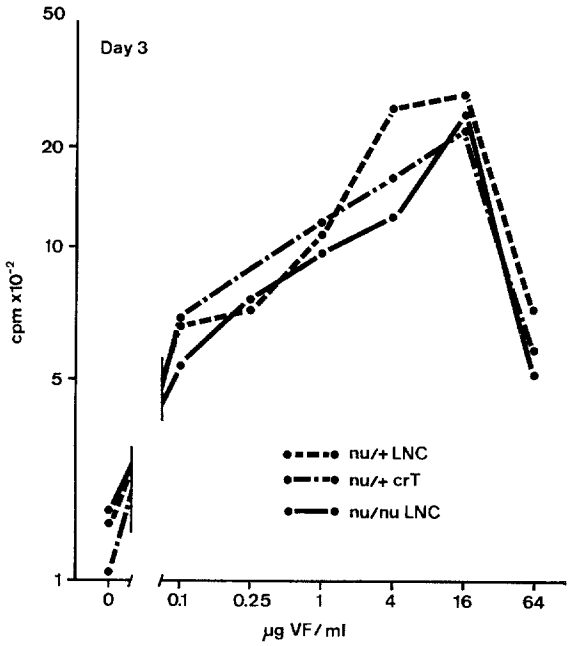

FIG. 2. [^{3}H]T incorporation into lymphocytes stimulated with graded doses of VF (cpm per culture day 3). Symbols represent arithmetic means of triplicate cultures.

$C3CF_1$ SC were preincubated with EDTA plasma from C5-deficient mice and SEPH-VFSF (mouse). The results suggest that SEPH-VF may be capable of conferring activated C3 onto C3 reactive lymphocytes.

However, if SEPH-VFSF and EDTA were omitted from the incubation mixture, fresh homologous serum by itself was found to completely suppress the rosette-forming ability of mouse and GP CRL. It was therefore interesting to determine whether this phenomenon was also complement dependent. $C3CF_1$ SC were incubated with differently pretreated sera from the same mouse strain (final concentration 1:5), washed extensively, counted, checked for viability, diluted, and then exposed to EAC. The results summarized in Table II demonstrate that the inhibitory effect of homologous serum was abolished by the addition of 10 mM EDTA, or by heat inactivation (56°C, 30 min) or zymosan absorption. Likewise, pretreatment of the donors with VF (total dose 250 U/kg) 48 h before serum collection completely suppressed the blocking capacity of mouse serum. Injection of donor mice with heat-inactivated (65°C, 30 min) VF was ineffective (data not shown in Table II).

Similar results[4] were obtained with SC and LNC from various mouse strains, including nu/nu lymphoid cells, and with SC and LNC from GP. Serum from

[4] Dukor, P., H. Probst, and D. Bitter-Suermann. Manuscript in preparation.

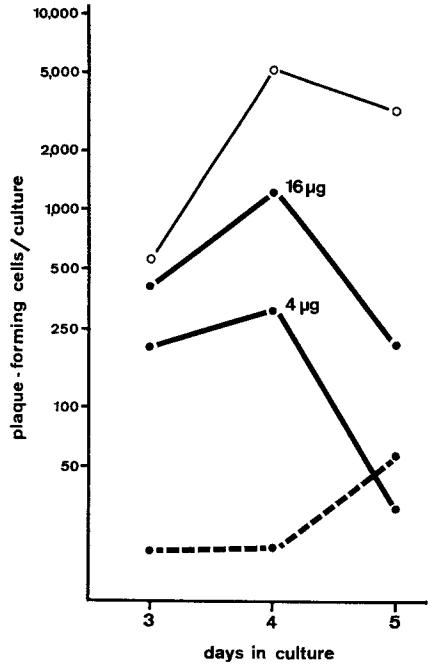

FIG. 3. Reconstitution by VF of the 19S PFC response to SRBC in T-cell-deprived SC cultures. O—O: 1.5 × 10^7 nu/+ SC; (●—●) 1.5 × 10^7 nu/nu SC with 4 or 16 μg of VF; (●- -●) 1.5 × 10^7 nu/nu SC without VF. Symbols represent arithmetic means of triplicate cultures.

C5-deficient B10.D2-old/Sn mice was just as effective as sera from other donor strains. GP serum and FBS, on the other hand, failed to interfere with the rosette-forming potential of mouse lymphoid cells. However, the rosette-forming ability of GP lymphocytes was readily inhibited by GP serum. The results indicate that homologous serum is activated in vitro (by freshly prepared lymphoid cell suspensions) to block the C3 receptor of B lymphocytes, provided a zymosan-removable or VF-sensitive component, a heat-labile serum factor, and divalent cations are present. Consequently it seemed worthwhile to explore whether the proliferative capacity of B cells could be altered by pretreatment with homologous serum. Nu/nu LNC were incubated under aseptic conditions with different types of sera as described above, and cultured in the presence of different doses of Con A, an otherwise T-cell-specific mitogen (22). As is shown in Table III, preincubation with fresh homologous serum resulted in a small, but definite increase in the proliferative response. Heat-inactivated or zymosan-absorbed serum, or serum from VF-treated mice proved ineffective.

The data are compatible with the idea that B cells can respond to a T-cell-specific mitogen, provided a serum component such as cleaved C3 is conferred

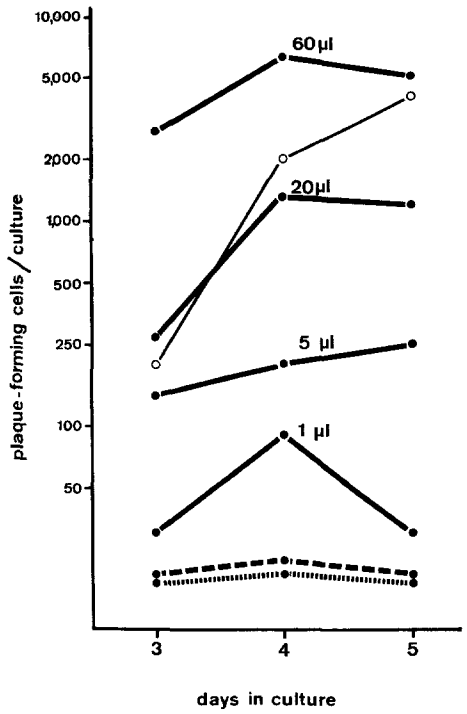

FIG. 4. Reconstitution by SEPH-VF of the 19S PFC response to SRBC in T-cell-deprived LNC cultures in the presence of 50 μM 2-ME. (●—●) 7×10^6 nu/nu LNC with graded doses of SEPH-VF; (○—○) 7×10^6 nu/nu LNC + 3×10^6 nu/+ crT without SEPH-VF; 7×10^6 nu/nu LNC without SEPH-VF; (● - - ●) 7×10^6 nu/nu LNC with 20 μl sepharose. Symbols represent arithmetic means of triplicate cultures.

to their C3 receptor sites by an additional mechanism. If this were true, it would also have to be postulated that B-cell activation in vitro might depend on a sufficient supply of exogenous complement components. In order to check this prediction, nu/+ crT, nu/+ LNC, and nu/nu LNC were cultured in the presence of native or hiFBS, or of zaFBS (C3 content 100, 70 and 8.3%, respectively). Although zaFBS did not interfere with the PHA-, VF-, or Con A-induced proliferation of T cells, it clearly failed to sustain the proliferative response of B cells to the B-cell activating mitogens PWM (22), LPS (23), VF, and SEPH-VF (Table IV). HiFBS, on the other hand, allowed B-cell proliferation to proceed normally. A selective toxicity of zymosan-absorbed serum for B cells appears unlikely, since cell recoveries were the same in both FBS- and zaFBS-supplemented nu/+ or nu/nu LNC cultures. Moreover, as is demonstrated in Table V, nu/nu SC, but not nu/nu LNC, could be readily stimulated by LPS in the presence of zaFBS.

These results suggest that a zymosan-removable serum factor is necessary

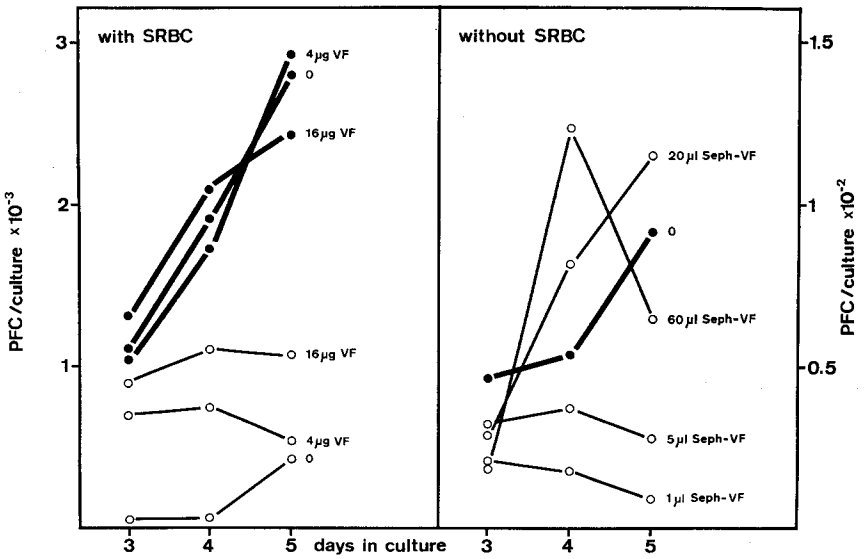

FIG. 5. Effect of VF and SEPH-VF on the 19S PFC response to SRBC in T-cell-sufficient and T-cell-deprived LNC cultures supplemented with 50 μM 2-ME in the presence or absence of antigen. Left side: cultures containing 3×10^6 SRBC. Right side: cultures without SRBC. (●—●) 7×10^6 nu/nu LNC + 3×10^6 nu/+ crT; (○—○) 7×10^6 nu/nu LNC. Addition of graded doses of VF or SEPH-VF as indicated in the figure. Symbols represent arithmetic means of triplicate cultures.

TABLE I

Inhibition of Rosette Formation by GP LNC Exposed to SEPH-VFSF, GP Serum, and EDTA

Preincubation treatment*	EAC rosette-forming cells,‡ exp. no.		
	1	2	3
	%	%	%
Control medium	24.5 ± 1.7	32.8 ± 4.1	31.0 ± 0
Seph-VFSF	20.5 ± 5.1	27.0 ± 2.4	
GP serum	19.5 ± 2.4	25.3 ± 3.6	28.6 ± 6.8
Seph-VFSF and GP serum	5.0 ± 1.0	10.3 ± 0.7	5.8 ± 1.7

* 30 min, 37°, 10 mM EDTA.

‡ Mean values of triplicates or quadruplicates ± SD.

for the mitogen-induced proliferation of B cells, and that this factor may be substituted by a constituent present in SC suspensions. Somewhat different results were obtained when the serum-dependence of antibody responses was investigated in a composite culture system (16): antibody formation to SRBC in vitro (Table VI) could be elicited only if native or heat-inactivated FBS was added to the medium. Anti-SRBC responses in the presence of either T cells or

TABLE II

Inhibition of Rosette Formation by $C3CF_1$ SC Exposed to Complement-Sufficient and Complement-Depleted Homologous Serum

Preincubation treatment*	EAC rosette-forming cells‡ and exp. no.		
	1	2	3
	%	%	%
Control medium	20.6 ± 1.2	18.0 ± 0	14.3 ± 1.0
Native mouse serum	0.7 ± 0.3	0.5 ± 0.3	0
Native mouse serum, 10 mM EDTA	22.4 ± 1.8		
Heat-inactivated mouse serum	20.6 ± 1.3		
Zymosan-absorbed mouse serum		21.3 ± 3.8	
Serum from VF-treated mice			14.3 ± 2.4

* 30 min, 37°.

‡ Mean values of triplicates or quadruplicates ± SD.

TABLE III

Proliferation of Serum-Preincubated B Cells (nu/nu LNC) Stimulated by Con A

Preincubation treatment*	Stimulation index day 3‡	
	Con A 1 μg/ml	Con A 4 μg/ml
Control medium	1.2 ± 0.1 (8)§	0.8 ± 0.1 (8)
Native mouse serum	5.9 ± 0.2 (10)	7.4 ± 0.1 (10)
Heat-inactivated mouse serum	1.2 ± 0.2 (3)	1.2 ± 0.8 (5)
Zymosan-absorbed mouse serum	1.1 ± 0.1 (4)	1.4 ± 0.8 (5)
Serum from VF-treated mice	1.1 ± 0.2 (3)	1.3 ± 0.2 (3)

* 30 min, 37°.

‡ [^{3}H]T incorporation: experimental/controls, mean values ± SE.

§ Number of triplicate cultures.

T-cell substituting mitogens (LPS, VF, SEPH-VF) did not occur in culture medium supplemented with zaFBS irrespective of the source of the B cells (SC or LNC) and the presence or absence of M.

DISCUSSION

The results presented in this communication demonstrate (*a*) that highly purified, pyrogen-free VF is strongly mitogenic for mouse T and B cells; (*b*) that VF, and even more effectively, SEPH-VF, restore the potential of antibody formation to SRBC in mouse B-cell cultures; (*c*) that SEPH-VF in the presence of complement may block the capacity of lymphocytes to form rosettes with

TABLE IV

Serum Requirements of Mitogen-Induced Lymphocyte Proliferation

Cells	Mitogen		[³H]T incorporation*; medium supplemented with:				
			Exp. 1			Exp. 2	
			8% FBS	8% hiFBS	8% zaFBS	8% FBS	8% zaFBS
	μg/ml		*cpm/culture day 3*				
Nu/+ crT	None		1,522		454	105	69
	PHA	16	31,784		21,393	12,457	11,594
	VF	16				3,042	3,738
Nu/+ LNC	None		1,341		988	320	265
	Con A	4	54,938		55,358		
	PHA	16	21,298		29,491	11,124	11,728
	VF	16				34,642	4,655
	LPS	16				7,805	682
Nu/nu LNC	None		563	205	321	615	241
	VF	16				9,730	388
	Seph-VF	20‡	3,254	1,862	349		
	LPS	16	24,634	18,330	546	8,688	701
	PWM	16	5,996	6,335	360		

* Mean values of triplicate cultures.

‡ μl suspension added per culture.

TABLE V

Serum Requirements of Mitogen-Induced B-Cell Proliferation in LNC and SC Cultures

Cells	Mitogen	[³H]T incorporation*; medium supplemented with:			
		Exp. 1		Exp. 2	
		8% FBS	8% zaFBS	8% FBS	8% zaFBS
	μg/ml	*cpm/culture day3*			
LNC nu/nu	None	358	278	563	321
	LPS 16	7,261	552	24,634	546
SC nu/nu	None	758	321	1,034	191
	LPS 16	24,638	5,468	24,310	3,268

* Mean values of triplicate cultures.

EAC; (*d*) that preincubation of freshly prepared B-cell suspensions with C3-sufficient, but not with C3-depleted homologous serum also inhibits their subsequent interaction with EAC and at the same time renders them reactive to an otherwise T-cell-specific mitogen (Con A); (*e*) that mitogen-induced B-cell proliferation and T-cell mediated or mitogen-triggered antibody formation against SRBC appear to depend on the availability of a serum factor which can be removed by zymosan; and (*f*) that in some culture systems this factor may

TABLE VI

Serum Requirements for Reconstitution by B-Cell Mitogens of T Cell-Deprived Cultures: PFC Response to SRBC

Cells	Mitogen		19S PFC/culture day 4*		
			Medium supplemented with:		
			10% FBS	10% hiFBS	10% zaFBS
		μg/ml			
Nu/+ SC‡	None		8,480	7,455	530
Nu/nu SC	None		45		
	LPS	4	1,340	945	185
	LPS	12	3,085	3,111	240
	VF	4	570	665	50
	VF	16	2,350	2,060	125
	SEPH-VF	40	7,240		180
M B	None		170		
M§ T‖ B¶	None		10,545	8,930	665
	LPS	12	2,875	2,340	245
	VF	16	1,210	1,005	90
B(+2=ME)**	None		85		
	SEPH-VF	40‡‡	6,360		415

* Mean values of triplicate cultures.
‡ 15×10^7 cells.
§ 3×10^5 nu/+M.
‖ 3×10^6 nu/+crT.
¶ 7×10^6 nu/nu LNC.
** 50 μM.
‡‡ μl suspension added per culture.

be substituted by a constituent present in SC suspensions. The interdependence of these phenomena is not yet clear. It is nevertheless tempting to speculate that they may be linked by a common mechanism and to interpret them in terms of the complement-hypothesis of B-cell activation (Fig. 6).

It is obvious that the signal for cell proliferation is provided by the way in which a mitogen interacts with the lymphocyte membrane. In this respect, mitogen-induced cell triggering is thought to represent a model for immunocyte activation by antigens. It has been postulated that different epitope densities are required for effective stimulation of T and B cells (24). It is also possible that antigenic determinants or the reactive groups of a mitogen have to be attached to a suitably rigid or poorly degradable backbone in order to become B-cell activators. Some support for the local concentration hypothesis of B-cell activation was furnished by the finding that locally concentrated mitogens, such as sepharose-coupled PHA (25), or Con A bound to a plastic surface (26),

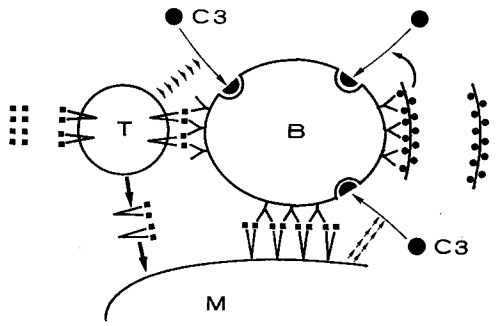

FIG. 6. Model of complement-mediated B-cell activation by T-dependent (■ ■) and T-independent (●●) antigens. B cells are triggered by the simultaneous or sequential generation of two signals, one antigen-related, one nonspecific. Generation of the specific signal depends on the appropriate interaction of the antigen with corresponding immunoglobulin receptors on the B cell membrane (Y), necessitating in the case of T-dependent antigens complexing with T-cell receptors (V) which may be released and bind to the surface of macrophages (M) (24). The nonspecific signal involves the binding of activated C3 (◒) to the C3 receptor (◡) of B lymphocytes. C3 (●) may be activated by T-independent antigens, or by nonspecific factors (proteases ?) released from antigen-stimulated T cells or macrophages (▲▲:, ▲) (9).

are capable of stimulating B cells, whereas the mitogenicity of the soluble material is restricted to T cells (22). On the other hand, a very different property appears actually to be shared by a number of representative B-cell activators: B-cell mitogens such as VF, LPS, PWM, and dextran sulfate (27), but not the T-cell-specific mitogens PHA and Con A, were found to be capable of initiating the alternative pathway of C3 activation. Similarly, T-independent antigens including Pneumococcus Type III polysaccharide, levan, polymerized flagellin, dinitrophenylated dextran, and, again, LPS were found to activate and convert C3 in C4-deficient GP serum, and to convert C3 and C3 proactivator in human serum.[2]

B-cell-activating molecules might therefore be able to confer cleaved C3 to the C3 receptors of the B cells to which they are bound, thus providing them with an essential nonspecific signal for triggering and/or proliferation. It is evident that not all bypass activators could be expected to be B-cell mitogens, since mitogenicity as such is likely to depend on suitable cross-linking of lymphocyte membrane components by the mitogenic agent. On the other hand, mitogens are known to interact with lymphocytes nonspecifically. The binding of mitogenic agents is therefore not restricted to the limited number of immunocytes bearing complementary immunoglobulin receptors. Hence, bypass-activating mitogens may also interact with B cells which at the same time bind an unrelated antigen. If this antigen is T dependent, i.e. incapable of activating C3 by itself, the bypass-inducing mitogens may now provide cleaved C3 required to initiate specific antibody formation. Indeed, as shown above, VF and SEPH-VF are able to substitute operationally for T cells in an otherwise pre-

dominantly T-dependent antibody response. Similar effects have been observed with PWM,[5] LPS (28), dextran sulfate (29), and polymerized flagellin (30). It is of course quite possible that the T-cell substituting effect of these agents is a direct consequence of their B-cell mitogenicity, as has recently been suggested by Sjöberg et al. (28). Moreover, M activation by B-cell mitogens or cross-reactivity between VF or PWM and SRBC have not yet been formally excluded.

Our results have further demonstrated that bypass activators may indeed be capable of conferring activated C3 to the C3 receptors of B cells: in the absence of divalent cations, preincubation of lymphocytes with homologous serum or plasma and SEPH-VFSF blocked their capacity to subsequently form rosettes with EAC 1423. In fact, preincubation of fresh lymphocyte suspensions with homologous serum in the presence of divalent cations was also found to inhibit rosette formation completely. Similar observations (release of cell-bound antigen-antibody complement complexes in the presence of serum) were recently made by Miller and Nussenzweig (31). Competitive blocking of the C3 receptor in these systems seems to depend on a factor from freshly prepared (excited?) lymphocytes (or macrophages), on divalent cations, on a heat-labile factor which was shown to be C3 proactivator (31), and on a zymosan-removable and VF-sensitive component, most likely C3. In the presence of all these required factors, C3 may be activated and conferred to the C3 receptor site of CRL. Indeed, "blocked" cells can be agglutinated by antimouse-β_{1C}, indicating the presence of C3 on their membranes[4]. Rosette inhibition may thus prove to be a useful tool for the detection of cell-associated mouse complement components.

The increased responsiveness of serum-pretreated B cells to stimulation with Con A, which was also demonstrated in this communication, is reminiscent of the acquisition of Con A reactivity by B cells in the presence of a supernatant factor from T-cell cultures (32). Since it is possible that activated T cells or macrophages may release a C3 cleaving enzyme (9), both sets of data are compatible with the idea that B cells can respond to a T-cell-specific mitogen, provided cleaved C3 is conferred to the complement receptor of CRL by an additional mechanism.

Finally, we have shown that mitogen-induced B-cell activation in LNC cultures and antibody formation to SRBC in composite cultures containing T cells or T-cell-substituting mitogens takes place only in the presence of normal or heat-inactivated, but not of zymosan-absorbed serum. Recent observations indicate that the same is true for the T-independent antibody response to dinitrophenylated dextran.[5] The C3 content of the untreated or heat-inactivated FBS correlates well with the proposed C3 requirements of B-cell activation by VF, PWM, and LPS. The strongly reduced C3 content of the zymosan-absorbed serum and its failure to permit B-cell proliferation and antibody

[5] Gisler, R. H., G. Schumann, and P. Dukor, unpublished observation.

production would also fit into the picture, even though it should be noted that absorption with zymosan removes not only C3 but various other serum factors involved in the bypass activation of the complement system. On the other hand, at least the C3 proactivator was certainly destroyed by heating the sera (33) and thus the bypass activation of C3 should not take place according to the data typically obtained with the conventional hemolytic assay for C3. It therefore remains to be explained why the presumed C3 proactivator-depleted serum still allows B-cell activation to occur. The following possibilities might be considered: (*a*) the cellular system is very sensitive and can be triggered already by a degree of bypass activation which can hardly be detected by a hemolytic assay for C3 turnover, (*b*) the cells replenish the system with the needed factors either by release or by having them absorbed to their surface, or (*c*) the proliferation and antibody formation induced by VF and LPS do *not* depend on the cofactors as defined for the bypass in vitro but rely on a yet unknown reaction mechanism which also leads to activation of C3. These possibilities are now under investigation.

Our findings indicate further that the requirement for an exogenous supply of zymosan-removable serum factors (C3?) is by no means absolute, since in SC cultures mitogen-induced B-cell proliferation was sustained by zaFBS. Indeed, using different culture conditions, SC responded to stimulation with LPS even in the total absence of serum (34). Mitogen-induced B-cell proliferation and polyclonal antibody formation in serum-free SC cultures was also observed by Coutinho et al. (35). It may be pertinent in this context that SC suspensions are known to contain a much greater proportion of macrophages than LNC (16), and that macrophages have been reported to synthesize or to carry complement components including C3 (36). In fact, recent evidence from our laboratory suggests that nonadherent SC can be stimulated by LPS only in the presence of serum, whereas mitogenic B-cell activation in unfractionated SC cultures is serum independent.[5] It will have to be explained, nevertheless, why in our systems zaFBS consistently failed to support antibody formation in vitro, irrespective of the presence of adherent SC or of peritoneal macrophages.

Preliminary data indicate that culture tube-adsorbed serum components may very effectively substitute for fluid phase serum factors (34). It might be possible, therefore, that under physiological conditions cell surface-associated complement components play an important role in cell triggering. This may be relevant to the observation that in vivo C3 depletion by VF in mice was found to inhibit T-dependent, but not T-independent antibody formation (21), and that a patient suffering from homozygous C3 deficiency had apparently normal serum-immunoglobulin levels (37). Although the data presented in this communication do not directly prove any of the assumptions of the complement hypothesis of B-cell activation, they nevertheless appear to be compatible with it and encourage further experimentation.

SUMMARY

It has been proposed that two distinct signals are required for the triggering of the precursors of antibody-forming bone marrow-derived cells (B cells): (*a*) the binding of antigen or of a mitogen to the corresponding receptor sites on B-cell membranes and (*b*) the interaction of activated C3 with the C3 receptor of B lymphocytes. There is growing evidence that B-cell mitogens and T (thymus-derived cell)-independent antigens are capable of activating the alternate pathway of the complement system (bypass). Therefore, the effect of another potent bypass inducer was investigated with regard to B-cell activation and the role of C3. Purified, pyrogen-free cobra venom factor was mitogenic for both T and B lymphocytes (cortisone-resistant mouse thymus cells and lymph node lymphocytes from congenitally athymic mice). Venom factor could substitute for T cells by restoring the potential of antibody formation to sheep red blood cells in mouse B-cell cultures supplemented with macrophages or 2-mercapto-ethanol. Venom factor may be capable of conferring activated C3 to the C3 receptor of B lymphocytes: preincubation of lymphoid cells with homologous serum or plasma, 10 mM EDTA, and sepharose-coupled venom factor converted with serum to an enzyme active against C3, inhibited their capacity to subsequently form rosettes with sheep erythrocytes sensitized with amboceptor and C5-deficient mouse complement. In the absence of EDTA, preincubation of freshly prepared B-cell suspensions with C3-sufficient homologous serum also blocked their subsequent interaction with complement-sensitized erythrocytes and at the same time rendered them reactive to an otherwise T-cell-specific mitogen. Moreover, mitogen induced B-cell proliferation in lymph node (but not in spleen) cell cultures, appeared to depend on the availability of exogenous C3: zymosan-absorbed fetal bovine serum (only 8.3% site-forming units remaining) supported T-cell activation by phytohemagglutinin, concanavalin A, and venom factor, but failed to sustain B-cell stimulation by pokeweed mitogen, lipopolysaccharide, and venom factor. T-cell-dependent antibody formation in composite cultures containing T cells or T-cell-substituting B-cell mitogens, B cells, and macrophages, always required the presence of C3-sufficient serum.

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