

## EFFECT OF B-CELL MITOGENS ON LYMPHOCYTE SUBPOPULATIONS POSSESSING C'3 AND Fc RECEPTORS\*

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It is well established that lymphoid cells possess receptors for the Fc fragment of antibody molecules complexed to antigen (Fc receptors) as well as for antigen-antibody-complement complexes (C'3 receptors) (1, 2). The presence of both Fc and C'3 receptors characterize bone-marrow-derived (B) cells and monocyte-macrophage cells (3). Recent findings suggest that the same B-lymphocyte population carry both Fc and C'3 receptors, because physical depletion of C'3-binding cells also removed Fc-binding cells (4).

The biological function of B cells carrying Fc and C'3 receptors is to a large extent unknown. So far, only one functional property has been clearly ascribed to cells having Fc receptors, namely the ability to be triggered in vitro to exert cell-mediated cytotoxicity when confronted with antibody-coated target cells (5-10). Recent studies on the nature of the cytotoxic effector cells against antibody-coated target cells (11-18) showed that B lymphocytes were most likely the cytotoxic effector cells, although a divergent opinion has also been expressed (19, 20). It was previously reported (16) that spleen cells prestimulated with B-cell mitogens, such as lipopolysaccharides (LPS)<sup>1</sup> from *Escherichia coli* bacteria and pneumococcal polysaccharide type SIII (SIII), markedly increased the efficiency of B-cell cytotoxicity against antibody-coated targets. The conclusion that the cells were B lymphocytes was based on the findings that they all possessed surface Ig, transformed morphologically and exhibited mitosis after LPS treatment, carried Fc receptors, and did not adhere to plastic surfaces or to iron particles (16). In addition about 40% of the LPS-activated blast cells were high rate Ig-producing cells (16). However, a third B-cell mitogen (purified-protein derivative of tuberculin [PPD]) was not able to increase the cytotoxic efficiency, even though LPS, SIII, and PPD were identical with regard to induction of polyclonal antibody synthesis, increased rate of DNA synthesis, and efficiency in transforming B cells into blast cells (16). Therefore, these findings suggested that LPS and SIII on the one hand and PPD on the other activated different types of B cells. The existence of two types of B cells could be clearly demonstrated after mitogen

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<sup>1</sup> Abbreviations used in this paper: BSS, balanced salt solution; FITC, fluorescein-isothiocyanate; LPS, lipopolysaccharides from *Escherichia coli* bacteria; PPD, purified-protein derivative of tuberculin; RFC, rosette-forming cells; SIII, pneumococcal polysaccharide type SIII; SRBC, sheep red blood cells.

activation (16): One was transformed into blast cells and had a high rate of Ig production whereas another was transformed into blasts but did not synthesize Ig. However, LPS and PPD had the same effect on these two types of B cells.

In order to determine whether different B-cell mitogens would have a selective effect on different B lymphocytes, we studied the effect of the three above-mentioned mitogens on cells capable of binding Fc receptors and C'3 receptors. The aims of the experiments were (a) to determine whether various B-cell mitogens acted selectively on different B-cell subpopulations and (b) whether there would be subpopulations of B cells capable of binding either Fc or C'3 or both. In order to approach these questions it was necessary to develop a method allowing the simultaneous detection of cells binding Fc and C'3 receptors. It will be shown that it is possible to detect whether individual lymphocytes carry receptors for both Fc and C'3 and that B-cell mitogens selectively activate lymphocytes in culture which are different with regard to the type of receptor they carry.

#### *Materials and Methods*

*Mice.*—Mice of the inbred strains A, 5M, CBA, C57BL and F<sub>1</sub> hybrids between these strains were used. In addition nude mice on Balb/c background were purchased from Bomholtgaard, Rye, Denmark.

*Mitogens.*—LPS of *E. coli* O55:B5 was prepared by phenol-water extraction (Dr. T. Holme, Department of Bacteriology, Karolinska Institutet, Stockholm). Before use in culture, the lyophilized preparation was dissolved in balanced salt solution (BSS) (1 mg/ml). SIII was obtained from The Wellcome Foundation, Ltd., Wellcome Research Laboratories, Kent, England (lot K 5045) and was dissolved in BSS to the desired concentration. PPD was obtained from the State Serum Institute, Copenhagen, Denmark, as a sterile solution containing 1 mg/ml in preservative free medium.

*Antisera.*—Antisera for the elaboration of Fc rosette-forming cells (RFC) was obtained from a rabbit after 6 weekly intravenous injections of 1 ml of a 25% suspension of sheep red blood cells (SRBC). The serum was separated on a Sephadex G-200 column (Pharmacia Fine Chemicals, Inc., Upsala, Sweden) and the 7S fraction was collected and concentrated to original serum volume by negative pressure dialysis. A 19S fraction from a rabbit anti-SRBC antiserum was obtained from Dr. Knut Lidman, National Bacteriology Laboratory, Stockholm, Sweden. It was isolated by Sephadex G-200 chromatography and had an agglutinin titer of  $\frac{1}{1,280}$ . It was used at a final concentration of  $\frac{1}{64}$ . In two experiments, a commercial amboceptor (Flow Laboratories, Irvine, Scotland) was employed. This antiserum had a hemolytic titer of  $\frac{1}{8,000}$  and an agglutinating titer of  $\frac{1}{256}$ . After 2-mercaptoethanol treatment the hemolytic titer was less than  $\frac{1}{2}$  and the agglutinating titer  $\frac{1}{32}$ . It was used at a final dilution of  $\frac{1}{500}$ .

*Determination of Fc-Binding Lymphocytes.*—In order to study the number of lymphocytes capable of binding to the Fc portion of antibody molecules, the following procedure of Bianco et al. (1) was followed in principle. 1 ml of SRBC (1%) was mixed with the 7S fraction of a rabbit anti-SRBC antibody so that the final antiserum dilution was  $\frac{1}{100}$  and incubated for 30 min at room temperature. Thereafter, the suspension was cooled in ice and washed three times in the cold. 0.1 ml of a 1% suspension of the antibody-coated SRBC was added to 0.1 ml of a suspension of lymphocytes ( $25 \times 10^6$  cells/ml) and finally 0.5 ml BSS was admixed. The cells were centrifuged at 750 rpm in the cold and carefully resuspended by a pasteur pipette, and the number of Fc rosettes was determined in the microscope.

*Determination of C'3-Binding Lymphocytes.*—Two different antisera were employed as outlined before. In short, the following procedure was followed. 1 ml of a 1% SRBC solution was treated with a rabbit anti-SRBC IgM preparation at a final dilution of  $\frac{1}{640}$  (or  $\frac{1}{500}$  with the second serum) for 30 min at 37°C. The SRBC were then washed in the cold three times and resuspended to the original volume. Subsequently, fresh strain A mouse serum as a source of complement, was added to the suspension at a final dilution of  $\frac{1}{5}$ . The suspension was incubated for another 30 min at 37°C, and then the SRBC were again washed three times and diluted to 1% in BSS.

Both antisera employed were tested for their ability to cause RFC formation in the absence of added mouse sera as a source of complement. The first antiserum (column separated 19S) did not cause any RFC to form in the absence of complement, whereas about 25% of the number of RFC detected in the presence of complement were formed with the commercial antiserum. The rosette assay was identical to that described above.

*Labeling of SRBC with Fluorescein-Isothiocyanate (FITC).*—5 mg FITC (Sigma Chemical Co., St. Louis, Mo.) was dissolved in 1 ml 0.12 M bicarbonate buffer pH 9.23. 1 ml FITC solution was mixed with 0.5-ml packed and washed SRBC and the mixture incubated for 30 min at room temperature. After four washings in BSS the SRBC were used in the rosette assay for the detection of Fc- or C'3-binding cells.

*Simultaneous Detection of Fc- and C'3-Binding Cells.*—To achieve this, FITC-labeled SRBC were coated with the 7S rabbit anti-SRBC fraction and unlabeled SRBC were coated with C'3 (or the reverse which worked equally well). The two cell suspensions were mixed in a 1:1 ratio and the rosette assay was carried out. Reading of RFC was performed in a fluorescent microscope (Leitz equipped with Ploem illumination). The RFC were first localized in phase-contrast and thereafter ultraviolet light was used. It was decided whether all adhering red cells were FITC labeled or not, or whether there was a mixture of labeled and unlabeled red cells.

*Tissue Culture Methods.*—Spleen cells were cultivated in petri dishes (Nunclon, Nunc, Denmark) as described by Mishell and Dutton (21) except that the cultures were not supplemented with serum (22, 23). After 1–3 days, cells from six dishes in each group were pooled, washed twice, resuspended to 1 ml, and used in the rosette assay. The cell concentration used for the rosette assay varied between 20 and  $30 \times 10^6$  cells/ml.

## RESULTS

*Subpopulations of B Cells Having Fc and C'3 Receptors.*—In order to study whether individual B lymphocytes possess both Fc and C'3 receptors or only one of them it was necessary to develop a method making it possible to simultaneously detect the presence of receptor-carrying cells. This was achieved by using a rosette technique employing FITC-labeled and unlabeled SRBC. These two types of SRBC were sensitized with either the IgG fraction from a rabbit anti-SRBC antiserum (for the detection of Fc receptor-carrying cells) or with the 19S fraction of a rabbit anti-SRBC antiserum, which had reacted with SRBC in the presence of fresh mouse serum as a complement source (for the detection of C'3 receptor-possessing lymphocytes). FITC-labeled red cells sensitized with, for example, C'3 were mixed with an equal volume of unlabeled SRBC sensitized with Fc, and added to a lymphocyte suspension. The results are presented in Table I and in Figs. 1–6. As shown before (16), about 75–88% of spleen cells from nude mice possessed Fc receptors, whereas the proportion of C'3-binding cells was slightly lower. In normal mice, about 35% of the spleen

TABLE I  
Occurrence of Fc and C'3 RFC in mitogen-stimulated spleen cells

Mouse strain	Mitogen	Days in culture	% of the RFC possessing the indicated receptors			% RFC out of total cells	% of the cells exhibiting surface Ig*
			Fc	C'3	Both		
Nude	—	1	30	0	70	88	NC†
	LPS	1	41	0	59	73	“
	PPD	1	63	0	36	80	“
	—	3	30	0	70	79	“
	LPS	3	86	0	14	58	“
	PPD	3	96	0	4	54	“
Nude	—	2	40	0	60	82	93
	LPS	2	38	0	62	75	100
	PPD	2	88	6	6	75	93
	SIII	2	41	0	59	75	93
A × BL	—	2	29	7	64	NC	NC
	LPS	2	86	0	14	“	“
	PPD	2	79	0	21	“	“
	—	3	18	6	76	“	“
	LPS	3	94	0	6	“	“
	PPD	3	95	0	5	“	“
A × 5M	—	0	14	0	86	“	“
	—	1	24	8	68	“	“
	LPS	1	32	5	63	“	“
	PPD	1	67	5	29	“	“
	—	2	19	0	81	“	“
	LPS	2	64	0	36	“	“
	PPD	2	83	0	17	“	“
A × 5M	—	0	26	3	69	“	“
	LPS	1	45	0	55	“	“
	PPD	1	58	5	37	“	“
A × 5M	—	2	32	0	68	“	“
	LPS	2	31	0	69	“	“
	PPD	2	70	0	30	“	“
	SIII	2	25	0	75	“	“
A × 5M	—	0	29	0	71	“	“
	—	2	27	8	65	“	“
	LPS	2	40	0	60	“	“
	PPD	2	91	0	9	“	“
	SIII	2	29	0	71	“	“
A × 5M	—	2	36	0	64	“	“
	LPS	2	41	0	59	“	“
	PPD	2	83	0	17	“	“
	SIII	2	48	0	52	“	“

\* Performed on living cells in suspension as originally described by Möller (27).

† NC, not counted.

cells bound Fc-sensitized SRBC and about 25% bound C'3-coated SRBC. The most interesting finding was that not all cells carrying the Fc receptors could bind C'3. Furthermore, few if any cells possessed only C'3 receptors. Thus, about 70% of the RFC in normal and nude mice were capable of binding both Fc- and C'3-sensitized SRBC, whereas about 30% bound only Fc. These two populations could be easily visualized by the use of the FITC-labeled SRBC (Figs. 1-8). It was shown that FITC labeling did not interfere with the binding

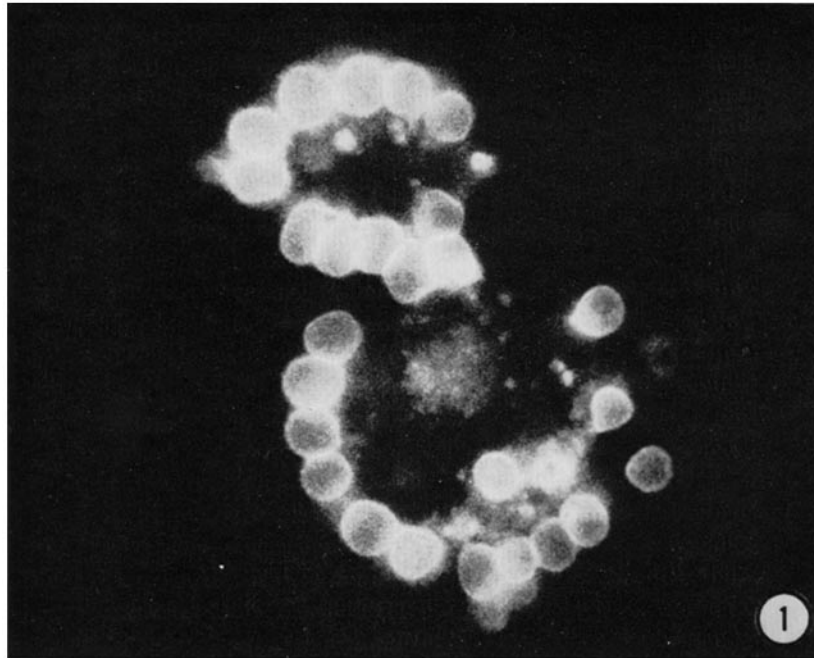


FIG. 1. Two RFC obtained from nude spleen cells and incubated with a 1:1 mixture of Fc-sensitized FITC-labeled SRBC and C'3-sensitized unlabeled SRBC. The RFC were photographed in a fluorescent microscope and show binding of Fc-sensitized SRBC.

of antibodies or complement. Similar results were obtained whether or not the C'3- or the Fc-sensitized SRBC were labeled with FITC. If mouse serum, as a source of complement, was omitted when the IgM antibody was employed, there were no RFC detected with the 19S antiserum used in all but two experiments.

*Effect of B-cell Mitogens on Fc and C'3 Receptor-Containing B Cells.*—As outlined in the introduction, indirect evidence suggests that LPS and SIII on the one hand and PPD on the other activated different populations of B cells, since only the first two mitogens were able to increase the cytotoxic effect of B cells against antibody- (Fc) coated target cells. The following experiments

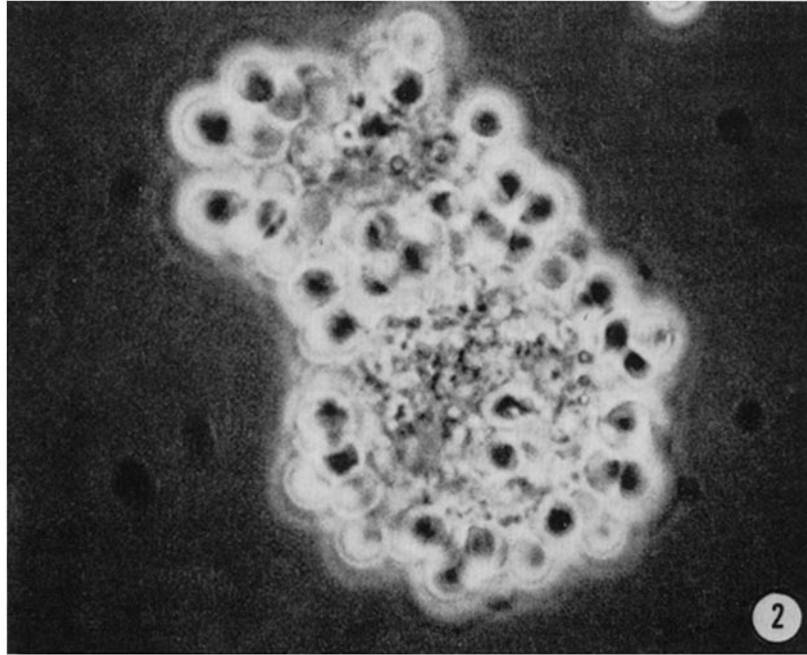
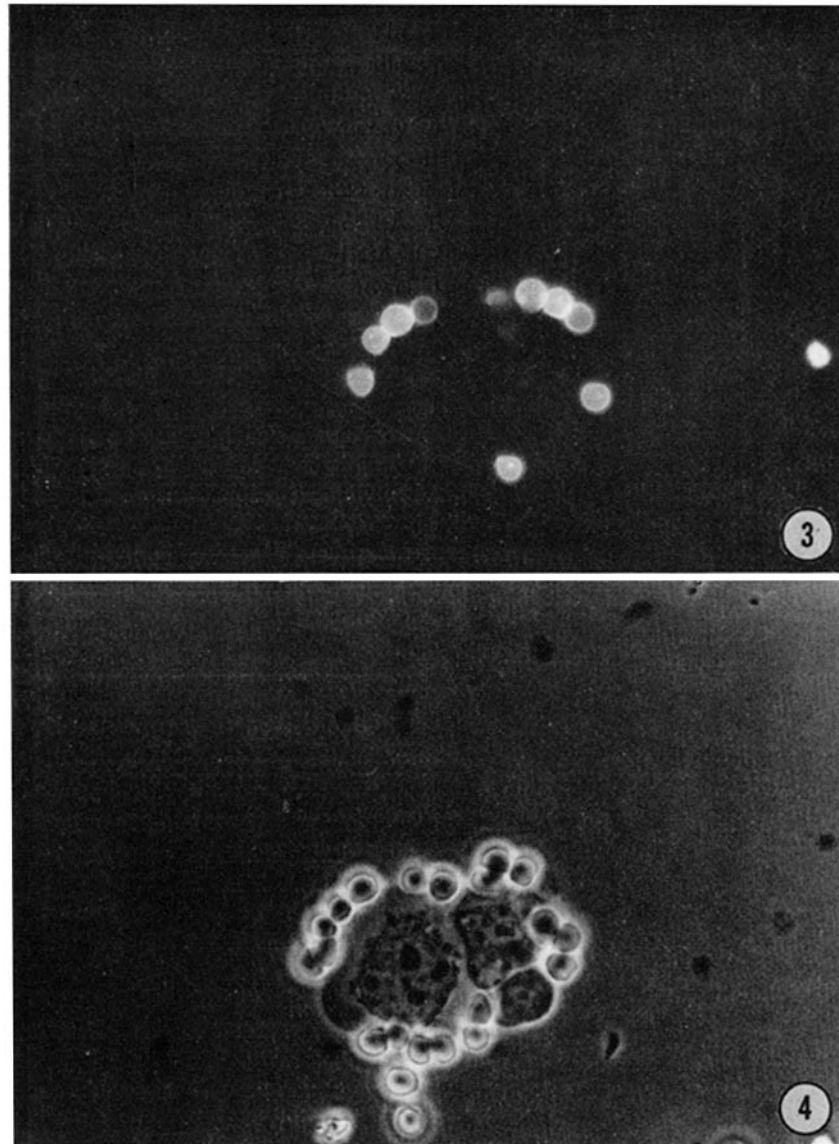


FIG. 2. The same cells as in Fig. 1 but photographed in phase-contrast. It can be seen that only a portion of the adhering red cells were labeled with FITC.

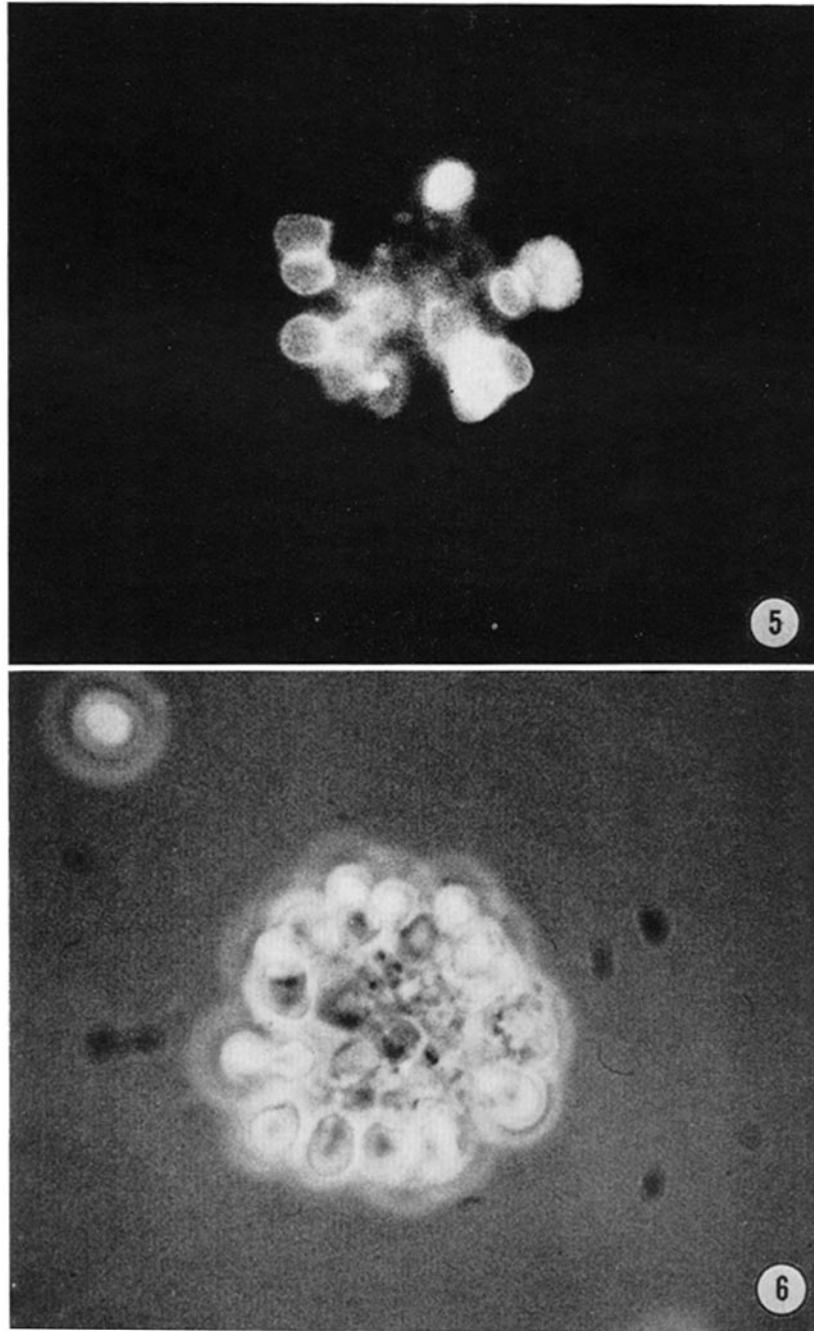
were performed in order to study whether the postulated subpopulations coincided with those capable of binding Fc- and C'3-coated SRBC: Normal mouse spleen cells or spleen cells from nude mice were cultured for 24 h with optimal concentrations (100  $\mu\text{g}/\text{ml}$ ) of the three B-cell mitogens mentioned. The cultures were not supplemented with serum, since induction of polyclonal antibody synthesis, DNA synthesis, and morphological transformation by mitogens does not require the presence of serum. After 24 h the cells were taken out and tested for the presence of Fc and C'3 receptors. As can be seen in Table I, LPS-stimulated cultures contained a large proportion of cells binding Fc as well as C'3-sensitized SRBC, whereas PPD-stimulated cultures had a much lower proportion of cells possessing receptors for both Fc and C'3. The proportion of cells binding Fc and C'3 in the LPS-stimulated cultures was approximately the same as in spleen cells from normal mice.

A kinetic study was performed in an analogous way. It was found that the frequency of Fc- and C'3-binding lymphocytes, stimulated by PPD, decreased rapidly after 2 and 3 days in culture (Table I, and Figs. 9 and 10) and after 3 days nearly all RFC only bound Fc-sensitized SRBC. Analogous findings were obtained with LPS-stimulated lymphocytes, although the decline in Fc-C'3-binding lymphocytes was much more marked between days 2 and 3. SIII gave results analogous to those obtained with LPS (Table I). It should be pointed



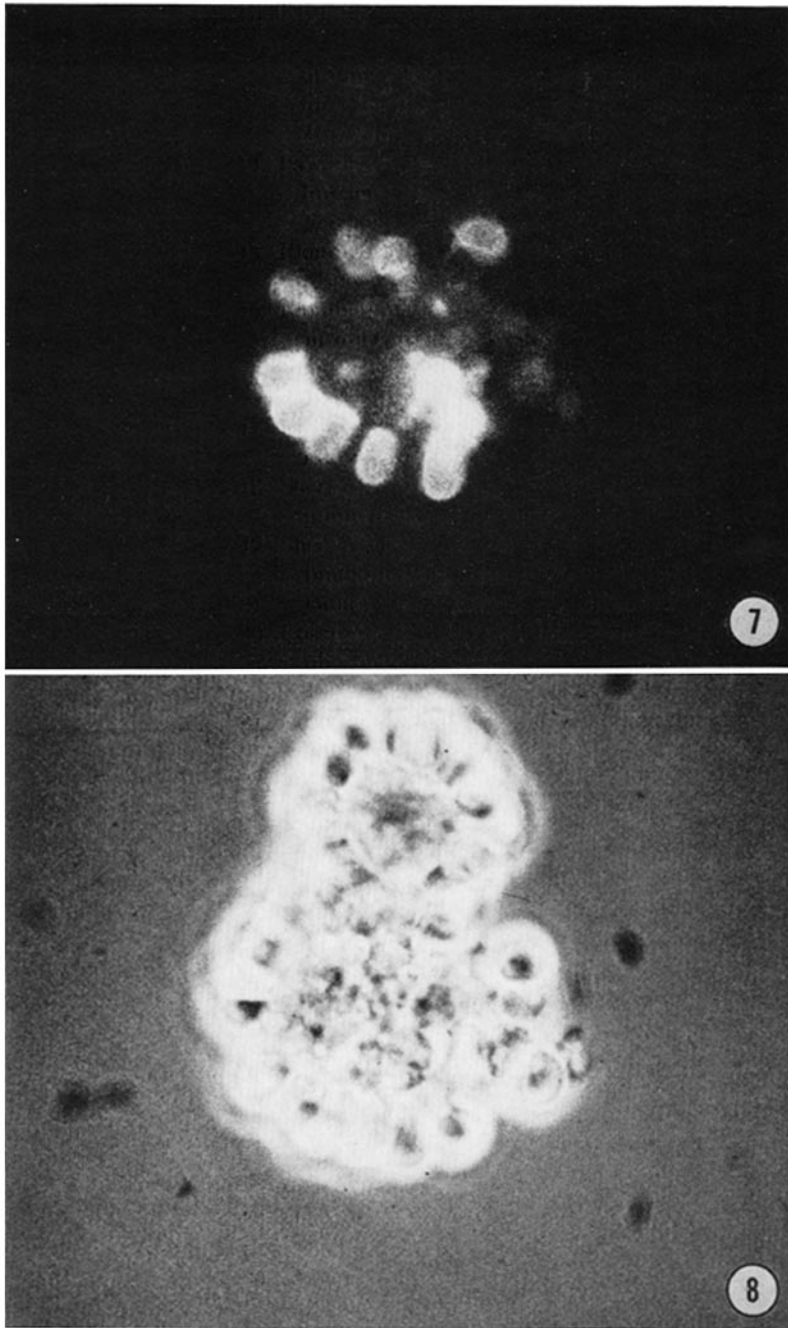
FIGS. 3 and 4. Same as Figs. 1 and 2, but the cells have been pressed between the cover glass and the slide to facilitate phase-contrast microscopy. Not all adhering red cells are FITC labeled (Fc sensitized).

out that the decline of Fc-C'3-binding cells was not caused by an ability of PPD or LPS to block the C'3 receptors, since pretreatment of fresh lymphocytes with these mitogens for 2 h at 37°C did not decrease the frequency of C'3-binding cells.



FIGS. 5 and 6. RFC with about half of the red cells being FITC labeled, Fc sensitized, and the other half unlabeled and C'3 sensitized.





FIGS. 7 and 8. C'3-sensitized FITC-labeled red cells mixed with Fc-sensitized unlabeled red cells. One RFC contains only Fc-binding cells, the other a mixture of Fc- and C'3-binding cells.

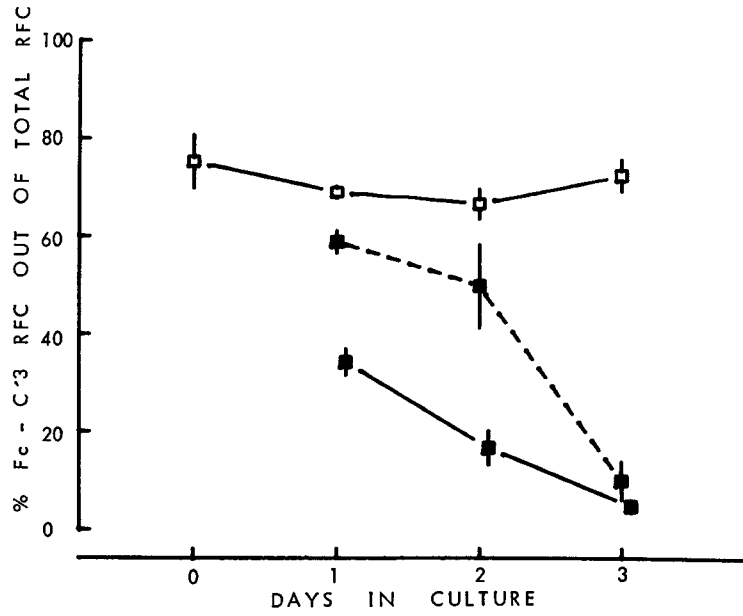


FIG. 9. Effect of B-cell mitogens on Fc-C'3-binding RFC. Spleen cells were cultivated in serum free medium in the absence of any mitogen (□-□) or were stimulated with 100  $\mu\text{g}/\text{ml}$  of LPS (■-■) or the same concentration of PPD (■-■).

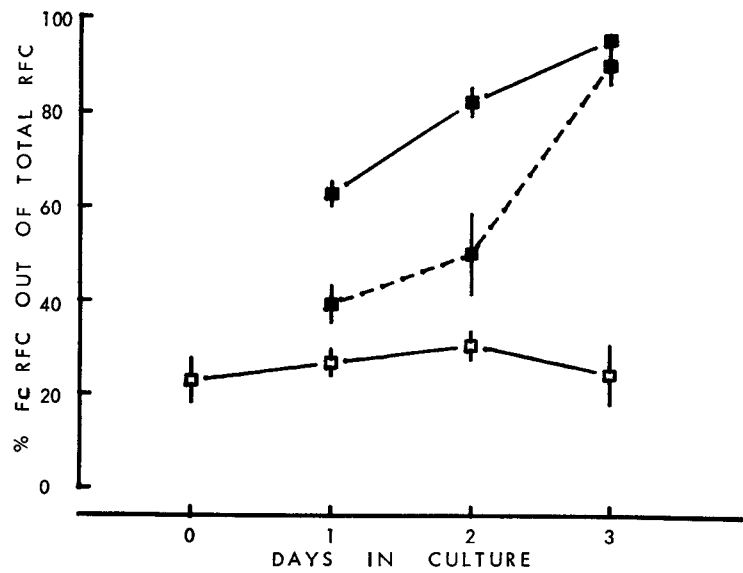


FIG. 10. Effect of B-cell mitogens on the Fc-binding RFC: Legend as in Fig. 9.

The proportion of blast cells and high rate immunoglobulin-synthesizing cells was previously found to be the same in LPS-, SIII-, and PPD-stimulated cultures (16). The present findings show that although these three B-cell mitogens induced morphological transformation and activated immunoglobulin synthesis to the same extent, they differed in their effect on cells having only Fc receptors or both Fc and C'3 receptors. Whereas LPS and SIII did not cause a major shift on Fc-C'3-binding lymphocytes after 24 h of stimulation, as compared to untreated cultures, PPD stimulation resulted in a large fall of Fc-C'3-binding cells and a corresponding increase of Fc-binding lymphocytes. However, if any of the B-cell mitogens used were allowed to interact with the spleen cells for 2-3 days, the lymphocytes capable of binding Fc-C'3 more or less completely disappeared.

#### DISCUSSION

These findings demonstrate the existence of a population of B cells bearing receptors for both Fc and C'3 and another possessing only Fc receptors. In addition, it was shown that different B-cell mitogens caused a change in the proportion of cells possessing Fc and C'3 receptors. The finding that there is a population of B cells which can bind both Fc and C'3 and another which can only bind Fc is in contrast to previous findings, suggesting that both receptors are present in the same B-cell population (4). It should be pointed out, however, that the method explained in this paper does not rely upon physical separation of the two cell types, but allows for a direct visualization of double-binding cells, and therefore, is less likely to be influenced by difficulties inherent in all physical separation methods, in particular when a small subpopulation is to be detected.

Previous studies (16) on B-cell cytotoxicity expressed against antibody-coated target cells strongly suggested the existence of subpopulations of B cells, because prestimulation for 24 h of normal lymphocytes by LPS and SIII, but not by PPD, markedly increased the degree of cytotoxicity. Since it was previously shown that LPS and PPS on the one hand and PPD on the other hand did not have any selective effect on the Fc-binding lymphocytes it seemed plausible that another surface receptor was involved. Since it has been shown that binding of lymphocytes to C'3-sensitized target cells does not result in cytotoxicity, whereas target cells sensitized with both Fc and C'3 are lysed to a greater extent than only Fc-coated target cells (24), it seemed likely that the subpopulation responsible was capable of binding C'3. Previous (15) as well as the present results are in accordance with this suggestion. It is noteworthy that LPS and SIII only activated cytotoxicity on antibody-coated target cells after prestimulation of the lymphocytes for 24 h, whereas there was a markedly depressed cytotoxic effect after prestimulation for 48 and 72 h. Thus, the decline of cytotoxicity paralleled the disappearance of Fc-C'3-binding lymphocytes.

The marked reduction of the Fc-C'3-binding population after PPD stimulation and after LPS activation for 48 and 72 h may have several explanations.

It may be a tissue culture artifact in the sense that nonstimulated lymphocytes do not survive well in culture. If LPS and SIII initially activate both the Fc-C'3- and the Fc-binding lymphocytes, whereas PPD only stimulates the Fc-binding lymphocytes, cultures treated with LPS and SIII for 24 h would show the same proportion of Fc-C'3- and Fc-binding cells as normal lymphocytes because both populations would survive, whereas only the Fc-binding cells would survive in the PPD-stimulated cultures. However, this explanation cannot adequately account for the rapid loss of C'3-binding cells in LPS cultures after 2 and 3 days. The possibility also exists that the Fc-C'3- and Fc-binding cells, respectively, are distinct populations and that the Fc-binding cells rapidly overgrow the other. However, this is not consistent with the rapidity of the change (24 h for PPD) which would not allow cell division to any major extent. Furthermore, the proportion of RFC out of the total number of cells actually declined with time in mitogen-treated cultures (Table I). It seems more likely, but cannot be proved as yet, that the loss of C'3 receptors is caused by a differentiation event. In terms of this concept, the nonactivated B lymphocytes are equipped with both Fc and C'3 receptors. After mitogen activation they differentiate into blast cells and lose the C'3 receptors in the process. PPD would be more potent to achieve this differentiation than LPS and SIII, or, alternatively and more likely, PPD activates more differentiated lymphocytes, whereas LPS only stimulates immature cells. In the latter case PPD would affect cells already differentiated and they would rapidly lose the C'3 receptors, whereas LPS-activated cells had to differentiate substantially before losing the C'3 receptors.

The role of the Fc-C'3-binding cells in antibody-induced cell-mediated cytotoxicity remains to be established. Since C'3-binding cells by themselves do not cause a cytotoxic effect in the absence of Fc binding (24) it seems likely that the simultaneous binding of B cells to an Fc- and C'3-coated target cell results in a more stable effector cell, target cell interaction. The Fc receptor is clearly responsible for triggering of cytotoxicity (9, 18, 25), but the C'3 receptor may have a role in increasing the stability of the binding between effector and target cells. This is probably important in culture, since close contact is necessary for expression of cytotoxicity (9). Whether this type of cytotoxicity operates *in vivo* is not settled as yet, but recent findings (26) cast doubt on its relevance *in vivo*. Thus, the fact that the presence of low concentrations of sera in culture destroys or inactivates C'3 receptors is difficult to reconcile with an active role of these receptors for a function *in vivo* in the presence of 100% serum.

#### SUMMARY

By the use of a rosette method allowing the detection at the cellular level of lymphocytes simultaneously binding Fc- and C'3-sensitized red cells it was found that about 70% of the rosette-forming cells from spleens of nude and normal mice possessed receptors for both Fc and C'3, whereas 30% only had Fc receptors. Very few, if any, lymphocytes possessed only C'3 receptors.

The B-cell mitogens, purified-protein derivative of tuberculin (PPD), lipopolysaccharide from *Escherichia coli* (LPS), and pneumococcal polysaccharide type SIII (SIII), induced marked changes of these receptor-bearing lymphocytes. PPD caused a rapid loss of cells capable of binding C'3 and a concomitant increase of only Fc-binding cells, which was detected after only 24 h. LPS and SIII induced analogous changes, but they were not detected until 48 h and were not complete until after 72 h. It is suggested that immature lymphocytes possess both Fc and C'3 receptors and lose the latter receptor upon differentiation induced by B-cell mitogens PPD. and LPS would affect different populations of B cells, PPD-activating cells that have already reached a higher differentiation stage, whereas LPS and SIII would activate more immature B cells.

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