IMMUNOGLOBULINS ON THE SURFACE OF THYMUS-DERIVED CELLS ENGAGED IN THE INITIATION OF A HUMORAL IMMUNE RESPONSE*

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Normal mouse spleen cells can be depleted of their ability to respond to erythrocyte antigens by treatment with rabbit antiserum against mouse kappa light chains and complement (1). This suppression is believed to occur by cytolysis of cells that bind the antiserum to light chains on their surfaces. This paper investigates the cell types and functions in the immune response that might be affected by this treatment. By using the in vitro response to hapten (2,4,6-trinitrophenyl [TNP]) coupled to an erythrocyte antigen (2), one can distinguish the roles of hapten-specific precursor cells and carrier-specific "helper" cells (3). It was found that the main victim of inactivation by rabbit antiserum against mouse kappa light chains and complement is the thymus-derived, carrier-specific helper cell. The significance of this with respect to the possibility of the presence of antibody-like receptors on the surface of thymus-derived cells is briefly discussed.

Materials and Methods

Cell suspensions were prepared from the spleens of 2–3-month old male and female BDF_1 mice (C57BL/6 ♀ × DBA/2 ♂). They were cultured in a modified Eagle's medium containing 5% fetal bovine serum for 3, 4, or 5 days in the presence of erythrocyte antigen (4). The immunogen used in culture was either sheep erythrocytes (SRBC)[1] or SRBC coupled with a trinitrophenyl hapten, (TNP-SRBC) (2). Spleen cells forming 19S antibody against SRBC were determined by a modification (4) of the plaque technique of Jerne et al. (5). Spleen cells forming antibody against TNP were assayed by plaque formation of horse erythrocytes (HRBC) coupled with TNP (TNP-HRBC) (6). Mice that were primed to SRBC were given 0.2 ml of a 0.5% suspension of SRBC (about 10^7 cells) intravenously, 3–7 days before sacrifice.

Sheep-specific thymus-derived spleen cells were prepared by injection of $2–4 \times 10^7$ normal BDF_1 thymus cells and 0.1 ml of 20% SRBC (2×10^8 cells) intravenously into syngeneic

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[1] *Abbreviations used in this paper:* HRBC, horse red blood cells; PFC, plaque-forming cells; SRBC, sheep red blood cells.

recipients immediately after irradiation with 1000 R. A second injection of 0.1 ml of 20% SRBC was sometimes given at day 4 after irradiation. Spleens were taken from these mice at 7 or 8 days after irradiation and injection of thymus cells. Bone marrow-derived spleen cells were prepared by injection of 2×10^7 normal BDF_1 bone marrow cells intravenously into syngeneic recipients after 1000 R irradiation. The spleens of these mice were taken at day 7 or 8.

Cells were irradiated in vitro in suspension with 1000 R. A cobalt-60 source was used for irradiation of cells and animals. Antisera against mouse kappa light chains were raised in rabbits against diethylaminoethyl (DEAE)-cellulose–purified urinary protein from mice bearing myeloma tumor MOPC 46(7). The specificities of the anti-kappa sera were tested in Ouchterlony plates, where lines of identity were formed against mouse gamma globulin (50% $[NH_4]_2SO_4$ precipitate of normal mouse serum) and purified light chains. Anti-kappa serum and complement treatment of spleen cells was as described previously (1). Mouse spleen cells were incubated at 12×10^7 cells/ml with anti-kappa serum at 0°C for 20 min. Guinea pig serum (C′) was added as a complement source and the cells were incubated for 30 min at 37°C. Cells were then washed once and resuspended in fresh medium for culture. The amounts used were: 0.5 ml spleen cells, 0.25 ml anti-kappa serum, 0.25 ml complement. Guinea pig serum used as a complement source was absorbed when necessary with mouse spleen and thymus cells until it was not cytotoxic to mouse cells or to culture responses. Cell viability was tested, after incubating cells at 37°C with the guinea pig serum, by trypan blue dye exclusion (8). It is important to note that the reaction of mouse spleen cells with anti-kappa serum was carried out in the cold. If spleen cells were treated with anti-kappa in the cold and then incubated for as little as 15 min at 37°C before the addition of guinea pig serum, the procedure no longer inactivated the in vitro response of the spleen cells.

Some rabbit sera were found to be cytotoxic to mouse thymus cells in the presence of complement. These sera could be absorbed with thymus cells until they were no longer cytotoxic (two or three times at 1:20 v/v packed cells to serum) without significantly affecting the anti-kappa activity (as determined by Ouchterlony precipitin lines) or the effect on spleen cell responses.

RESULTS

Effect of Anti-Kappa Sera on Cell Populations Present in the Mouse Spleen.— The in vitro response of mouse spleen cells to erythrocyte antigens can be irreversibly abolished by a pulse pretreatment of the cells with rabbit antiserum against mouse kappa light chains (anti-kappa serum) and complement. This inhibition can be prevented by preincubation of the anti-kappa serum with normal mouse serum (1), with partially purified mouse gamma globulin (50% $[NH_4]_2SO_4$ precipitate), or with purified kappa chains from myeloma urinary protein (Table I). Inhibition of the immune response is believed to be a consequence of the cytolysis of cells which specifically bind anti-kappa antibody to membrane-associated light chains on their surface. Subsequent experiments sought to investigate the cell functions that were eliminated after treatment of a spleen cell population with anti-kappa serum and complement. The in vitro hapten-carrier system, consisting of trinitrophenyl groups coupled on sheep erythrocytes (TNP-SRBC) (3), was used to distinguish thymus-derived, carrier-specific helper cell activity and bone marrow-derived hapten-specific antibody precursor activity.

In this system, normal spleen cell cultures, in the presence of TNP-SRBC as immunogen, show a greatly enhanced primary response to TNP if irradiated spleen cells from mice that have been immunized to SRBC are added (3 and Fig. 1). All anti-TNP plaque-forming cells (PFC) still come from normal spleen, since the primed irradiated population can generate no antibody-forming cells. Control cultures of irradiated primed cells give no PFC against TNP (last line

TABLE I

Specificity of Inhibition of Normal Spleen Culture Responses by Treatment of Cells with Rabbit Anti-Mouse Kappa Serum and Complement

Spleen cell treatment	Plaque-forming cells/culture	
Experiment A	Anti-TNP	Anti-SRBC
None	554	2176
C′ only	935	1860
anti-k + C′	18	0
anti-k (0.1 ml) + MGG (about 3 mg) + C′	849	2610
Experiment B		
None		—
C′ only		683
		939
anti-k (0.1 ml) + C′		0
anti-k (0.1 ml) + P4 (0.11 mg) + C′		2062
anti-k (0.1 ml) + P4 (0.31 mg) + C′		450
anti-k (0.1 ml) + P4* (0.10 mg) + C′		93
anti-k (0.1 ml) + P4* (0.25 mg) + C′		608

Anti-kappa serum was raised in rabbits against the purified kappa chain designated P4 (MOPC 46). Antiserum was neutralized, where indicated, by preincubation for 15 min at room temperature with partially purified mouse gamma globulin (MGG) (50% $[NH_4]_2SO_4$-precipitated normal mouse serum) (Exp. A), or with DEAE-cellulose column-purified kappa chains (P4). All cultures contained 12×10^6 spleen cells initially; 3×10^6 TNP-SRBC in Exp. A, or 3×10^7 SRBC in Exp. B were added at time 0. Responses are PFC/culture at day 4.

* A different light chain preparation than was used to immunize the rabbits.

of Table II, and III). The irradiated population is providing an excess of carrier-specific helper cells which enable stimulation of more anti-TNP precursors in the normal spleen population than is possible by the normal spleen's own SRBC-specific helper cells. The antigen specificity of preimmunization to carrier has been demonstrated (3).

There is increasing evidence that carrier-specific helper activity is a function of the thymus-derived cell population in the spleen. In an in vivo cell transfer system, Raff (9) has shown that the helper activity of carrier-primed spleen cells in an anti-hapten response is abolished by treatment of the carrier primed

population with antiserum against the mouse thymus cell antigen, theta, and complement. The hapten-primed precursor population is not affected by anti-θ treatment. Similar results have been obtained with the in vitro response to TNP erythrocytes (10). Anti-θ treatment of the carrier (erythrocyte) immune

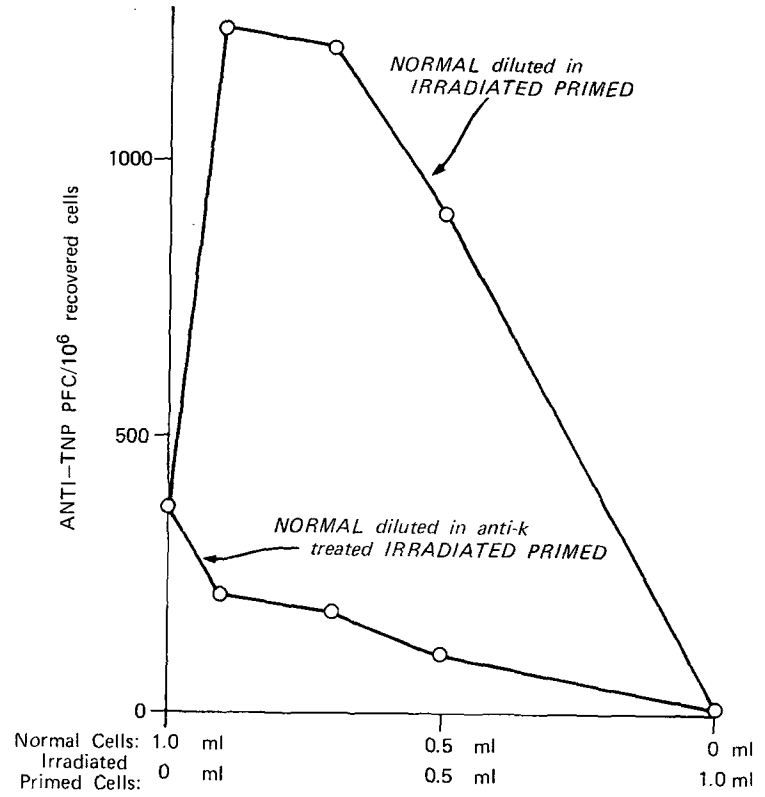

FIG. 1. Anti-kappa inhibition of irradiated primed spleen cell enhancement of normal spleen anti-TNP response. A total of 1.0 ml of cells at 12 $\times$ 10^6 cells/ml were cultured with TNP-SRBC. SRBC-primed spleen cells were from mice injected 3 days earlier with 0.2 ml of 0.5% SRBC. They were irradiated in vitro with 1000 R. Anti-kappa treatment of the irradiated, primed cells was as described in the text. Data from Fig. 3 of reference 10.

irradiated population abolishes its ability to enhance the anti-hapten response of normal spleen cultures.

The irradiated, SRBC-immune population was treated with anti-kappa and complement to determine the effect on the carrier-specific helper activity. The helper activity of the irradiated primed population can be completely abolished by a pulse treatment with anti-kappa serum and complement before mixing them with normal spleen (Fig. 1 and Table II). The fact that there is no in-

hibition of the normal spleen cell response argues against carry-over of anti-kappa antibody or nonspecific cytotoxic effects after anti-kappa treatment which might continue after the populations are mixed. The specificity of elimination of the carrier effect by anti-kappa serum was verified by the use of purified kappa light chains to block anti-kappa antibody. This prevented anti-kappa inhibition of the helper activity (Table II). Precipitating antigen-antibody complexes (mouse kappa chains, rabbit anti-kappa antibody) were

TABLE II

Blocking Anti-Kappa Inhibition of Carrier Enhancement with Purified Light Chains

	Anti-TNP responses (PFC/culture)		
	Day 3	Day 4	Day 5
Normal spleen	318	382	1489
Normal spleen + irradiated SRBC-primed	1552	4425	12,679
Normal spleen + anti-k–treated irradiated SRBC-primed spleen	384	537	2498
Normal spleen + P4-blocked anti-k–treated irradiated SRBC-primed spleen	1328	2664	13,420
Normal spleen + P4-blocked anti-K– and ALS-treated irradiated SRBC-primed spleen	—	—	2145
SRBC-primed spleen	2899	9413	29,972
Irradiated SRBC-primed spleen	2	0	0

1 ml of rabbit anti-mouse kappa serum was incubated with 0.4 mg purified light chain designated P4, to which the anti-serum was raised, overnight at 4°C. The precipitating material was sedimented and the neutralized serum was used to treat cells in the presence of complement. Irradiated, SRBC-primed cells were spleen cells from mice injected with 0.2 ml of 0.5% SRBC (about 10^7 cells) 3 days before sacrifice. Primed cells were irradiated in suspension with 1000 R and 6×10^6 were added/culture to normal spleen cells. Normal spleen cells, 6×10^6 cells/culture, were untreated. A rabbit anti-mouse lymphocyte serum (ALS) was also used, in the presence of P4-blocked anti-kappa + C′, to treat irradiated, SRBC-primed cells as verification of the availability of C′ for lysis. 3×10^6 TNP-SRBC were added at time 0. Values given are PFC recovered/culture on days 3, 4, and 5.

removed by centrifugation from the mixture of anti-kappa and kappa chains before the serum was added to cells with complement. In line 5 of Table II, rabbit anti-mouse lymphocyte serum was added along with the neutralized anti-kappa serum to verify that any antigen-antibody complexes remaining in the serum are not able to fix enough complement to prevent lysis when another antibody against mouse lymphocytes is present.

The effect of anti-kappa and complement treatment on precursors to antibody-forming cells supplied by the normal spleen was examined. It was found that normal spleen cells, rendered incapable of response to TNP or to SRBC by treatment with anti-kappa serum and complement could be restored by the

addition of irradiated primed cells (Table III). The anti-kappa–treated spleen must be supplying the precursors of the anti-TNP and anti-SRBC plaque-forming cells since the irradiated spleen cannot generate antibody-forming cells. Sometimes the restoration by irradiated primed cells can reach helper cell-enhanced levels of untreated cells, indicating that little damage has been done to the precursor population of the normal spleen by treatment with anti-kappa serum and complement. The variability in inhibition and restoration shown in this experiment (especially the last column, Table III) is representative of the variability seen among several experiments of this design. Irradiated normal

TABLE III

Restoration of Responses of Anti-Kappa–Treated Normal Spleen Cells by Irradiated, SRBC-Immune Spleen Cells

Normal spleen treatment	anti-TNP response (PFC/culture)		anti-SRBC response (PFC/culture)	
	Normal spleen alone	Normal spleen + irradiated SRBC-primed	Normal spleen alone	Normal spleen + irradiated SRBC-primed
None	334	3727	2363	4131
anti-k + C′	0	1220	2	180
	2	2550	28	650
anti-k* + C′	3	2469	38	2550
	12	2380	245	2200
Irradiated SRBC-primed	—	0	—	59

6×10^6 normal, unirradiated, spleen cells were present in all cases. 6×10^6 SRBC-primed irradiated spleen cells were added where indicated. Primed cells were obtained from the spleens of mice injected with 0.2 ml of 0.5% SRBC (about 10^7 cells) 3 days before culture. They were irradiated with 1000 R before culturing. 3×10^6 TNP-SRBC were added at time 0. Values given are PFC recovered/culture on day 4.

* Anti-kappa serum was used here at ½ the concentration normally used.

BDF_1 spleen cells do not restore anti-kappa–treated normal BDF_1 cells, indicating that immunization to the carrier of TNP is required.

Effect of Anti-Kappa Serum on Cell Populations Derived from the Thymus.— Mitchell and Miller (11) demonstrated the antigen requirement for preparation of thymus-derived cells in bone marrow-thymus cell interactions in irradiated, reconstituted mice responding to erythrocyte antigens. Vann and Kettman[2] (and see ref. 10) have found that carrier-primed thymus-derived spleen cells (from mice irradiated and injected with 3×10^7 thymus cells and SRBC 7 days before sacrifice) can substitute for irradiated SRBC immune spleen cells in the enhancement of the in vitro anti-TNP response of normal spleen cells cultured

[2] Vann, D. C., and J. Kettman. *In vitro* cooperation of cells of bone marrow and thymus origins in the production of antibodies. Manuscript in preparation.

in the presence of TNP-SRBC. Such a thymus-derived spleen population contains no precursors to antibody-forming cells and can make no immune response by itself. Its helper activity is not affected by irradiation.[2] Table IV shows the helper activity of SRBC-immune thymus-derived spleen cells in combination with normal spleen cells. The helper activity is eliminated by treatment of this population with anti-kappa serum and complement. Anti-kappa–depleted normal spleen can also be restored by SRBC-primed thymus-derived spleen cells.

TABLE IV

Enhancement of TNP Responses with Carrier-Primed Thymus-Derived Cells. Anti-Kappa plus Complement Sensitivity of Carrier-Primed Thymus-Derived Cells

Spleen cells in culture	Anti-TNP PFC/culture
Normal spleen	199
Normal spleen + Thymus-derived spleen (SRBC-primed)	2896
Normal spleen + anti-k + C′-treated thymus-derived spleen (SRBC-primed)	105
Anti-k + C′-treated normal spleen	30
Anti-k + C′-treated normal spleen + Thymus-derived spleen (SRBC-primed)	840
Anti-k + C′-treated normal spleen + anti-k + C′-treated thymus-derived spleen (SRBC-primed)	5
Thymus-derived spleen (SRBC-primed)	0

6×10^6 normal spleen cells or anti-kappa + complement-treated normal spleen cells were cultured with or without 6×10^6 thymus-derived cells primed to SRBC. Thymus-derived cells were spleen cells from mice lethally irradiated (1000 R) and injected with 3×10^7 normal thymus cells and 0.1 ml of 20% SRBC (about 2×10^8 cells) 7 days before culture. Where indicated, thymus-derived, SRBC-primed cells were treated with anti-kappa serum and complement. 3×10^6 TNP-SRBC were added at time 0. Values given are anti-TNP PFC recovered/culture on day 5.

Effect of Anti-Kappa Serum on Cell Populations Derived from the Bone Marrow.—Bone marrow-derived spleen cells can be prepared by injection of 2×10^7 normal bone marrow cells into lethally irradiated hosts 7 days before sacrifice. There is no antigen requirement in the preparation of these cells. Bone marrow-derived cells can be stimulated to generate anti-SRBC and anti-TNP responses in vitro if they are cultured with SRBC-educated thymus-derived cells or with irradiated SRBC-immune spleen cells (9). These cells were used to test further for anti-kappa sensitivity of precursors. Bone marrow-derived cells were treated with rabbit anti-mouse kappa serum and complement in the same way that normal spleen, irradiated primed spleen, and primed thymus-derived cells were treated previously. When treated and untreated bone marrow-derived spleen cells were cultured with irradiated, SRBC-immune spleen cells as a source of

helper cells, no depression in the response to TNP or to SRBC could be shown as a result of anti-kappa treatment (Table V). Thus, under conditions where thymus-derived spleen cells are sensitive to anti-kappa serum and complement, precursors are not sensitive. It is not known whether higher concentrations of antisera would have been inhibitory. It was noted that better restorations of the response of anti-SRBC precursors by irradiated primed cells were seen after treatment with a slightly lower anti-kappa concentration (last column, Table III).

TABLE V

Anti-TNP and Anti-SRBC Responses of Anti-Kappa-Treated and Untreated Bone Marrow-Derived Spleen Cells Diluted in Irradiated, SRBC-Primed Spleen Cells

Treatment of BM-derived spleen cells		Anti-TNP PFC/10^6 cells		Anti-SRBC PFC/10^6 cells	
		None	Anti-k + C′	None	Anti-k + C′
(*Cells cultured*)					
Irradiated SRBC-primed spleen		5	—	401	—
BM-derived spleen		7	0	2	6
BM-derived Spleen	+ Irradiated SRBC-Primed spleen				
0.7 ml	0.3 ml	5	0	2035	1437
0.5 ml	0.5 ml	63	123	4393	6710
0.3 ml	0.7 ml	34	211	6686	5922

A total of 1 ml of cells at 12 × 10^6 cells/ml were cultured with TNP-SRBC. Irradiated primed spleen cells were from mice injected 3 days earlier with 0.2 ml of 0.5% SRBC and irradiated with 1000 R. Bone marrow (BM)-derived cells were spleen cells from mice lethally irradiated (1000 R) and injected with 2 × 10^7 normal syngeneic bone marrow cells 7 days before culture. BM-derived spleen cells were treated with anti-mouse kappa serum by the same procedure used to treat normal, irradiated primed, and thymus-derived spleen cells. Anti-kappa + C′ treatment of normal spleens in concurrent experiments reduced responses by at least 100-fold. 3 × 10^6 TNP-SRBC were given at time 0. Values given are PFC/10^6 cells recovered on day 4.

DISCUSSION

It is clear that, in this system, the thymus-derived helper cells are rendered inactive, and are presumably lysed, by antiserum against mouse kappa chains and complement, and that the precursors to antibody-forming cells are relatively unaffected. Specific attempts to demonstrate inactivation of precursors with anti-kappa serum and complement in bone marrow-derived spleen populations were unsuccessful (see Table V). There may be some indication of damage to precursors in the failure in some cases (e.g. higher anti-kappa concentrations, as shown in Table III) to obtain complete restoration of anti-kappa and complement-treated normal spleen with carrier-primed helper cells (irradiated primed cells or carrier-educated thymus-derived cells). However, the data es-

tablish clearly different sensitivities of helper cell and precursor cell activity to anti-kappa serum and complement. (It is also of interest that mature plaque-forming cells are not sensitive to lysis by anti-kappa serum and complement, although the formation of IgM plaques can be inhibited by the presence of anti-kappa serum in the plaque assay. Since mature antibody-forming cells can be lysed by antisera against H-2 specificities, it must be concluded that these cells have sites for complement and membranes sensitive to lysis by antibody and complement.)

The results of treatment of spleen cells with rabbit anti-mouse kappa chain serum and complement before culture resemble those obtained upon treatment of mouse spleen cells with homologous antisera against the mouse thymus cell antigen θ (9 and 10). We take these experiments as strong evidence for the presence of immunoglobulin-like molecules, consisting at least of light chains of the kappa class, on the surfaces of thymus-derived cells. Since thymus-derived populations which are involved in cell cooperation in thymus-dependent humoral responses are antigen specific (11), it would be reasonable to postulate that the cell surface light chains detected here are involved in antigen recognition by helper cells.

There is other evidence suggestive of immunoglobulin-like receptors on thymus-derived lymphocytes. Greaves, Torrigiani, and Roitt (12) were able to inhibit delayed hypersensitivity and homograft reactions in cultures of human lymphocytes with antisera against human light chains. Mason and Warner (13) found inhibition of delayed hypersensitivity and graft-*versus*-host reactions in irradiated mice reconstituted with lymphocytes that had been preincubated in antisera against mouse light chains. They found no inhibition with antisera against mouse heavy chain classes. These primarily cellular immune responses are thought to be functions of thymus-derived lymphocytes. Inhibition of these responses by anti-light chain antibody is believed to occur by blocking of antigen-specific cell surface immunoglobulin-like receptors. Humoral immune responses can also be inhibited by the presence of antisera against mouse immunoglobulins in vitro (1 and 14) and in vivo (15), but here it is not clear what cell type is being blocked by the antisera, and antisera against other than light chain immunoglobulin components are effective. Greaves, Möller, and Möller (16) have found evidence for light chains on anti-θ–serum sensitive-, and therefore presumably thymus-derived, mouse rosette-forming lymphocytes, since the binding of sheep red blood cell antigen to these cells is inhibited by anti-mouse light chain sera, but not by anti-heavy chain sera.

Contrary to these indications of immunoglobulin components on the surface of thymus-derived cells is Raff's finding (17) that cells staining with fluorescent anti-mouse immunoglobulin antibodies are a distinct population from cells stained by anti-θ–serum. Since the anti-θ–staining population cannot be labeled independently, a certain amount of overlap might not be discernible. Alterna-

tively, immunoglobulin may be only acquired by thymus-derived cells as theta is diminished during maturation after leaving the thymus, in which case the sensitivity of the fluorescent technique may not be adequate to detect small amounts of immunoglobulin on theta-staining cells, or small amounts of theta on heavily immunoglobulin-staining cells.

It is perhaps interesting to note that in our hands the sensitivity to complement is lost when anti-kappa–treated cells are incubated in the warm for 15 min in the absence of free anti-kappa. The mechanism of this effect is not clear, but it does not seem to be due to dissociation of the antigen-antibody complexes on the cell surface. It is possible that minor differences in technique can account for conflicting observations.

If thymus-derived cells which have immunoglobulin components on their surfaces can be lysed by anti-mouse kappa sera, why are precursors to antibody-forming cells, which we also believe to possess antigen-sensitive, immunoglobulin-like receptors, not also inactivated by antisera against mouse immunoglobulin components? It is possible that membrane-bound immunoglobulin is associated with cell membranes in different ways in different cell types and at various stages of maturation. Precursor cell immunoglobulin could be attached or be distributed in such a way that the cells are not vulnerable to complement-mediated lysis. The invulnerability of precursor cell function, however, is not evidence that precursors do not have membrane-associated immunoglobulin receptors for antigen. In preliminary studies to be reported shortly, we have found that anti-kappa serum prevents the induction of an unresponsive state which occurs when bone marrow-derived cells are incubated with polyvalent hapten on a nonimmunogenic carrier.[3] The vulnerability of thymus-derived cell helper function is evidence for the existence of immunoglobulin molecules on the surfaces of these cells.

SUMMARY

Preculture treatment of normal spleen cells with antiserum against mouse kappa light chains and complement was found to inhibit in vitro responses of these cells to TNP and erythrocyte (carrier) antigens, primarily by elimination of a thymus-derived helper component required for the response. Spleen populations inactivated in this way could be reconstituted with irradiated, carrier-immune spleen cells or with carrier-educated thymus-derived spleen cells. The ability of helper populations (i.e. irradiated, carrier-immune spleen cells or carrier-educated thymus-derived spleen cells) to enhance the response of normal spleen cells to hapten was eliminated by pretreatment of the helper cells with anti-kappa serum and complement. No significant effect of anti-kappa and

[3] Kettman, J., and J. Lesley. Induction *in vitro* of specific unresponsiveness against the 2,4,6-trinitrophenyl determinant. II. Prevention and reversal of the unresponsive state. Manuscript in preparation.

complement treatment on precursor cell populations in normal spleen or bone-marrow-derived spleen could be demonstrated. The data are interpreted as evidence for the presence of immunoglobulin components. The function of these molecules is not established but it would be reasonable to assume that they are involved in antigen recognition, on the surface of thymus-derived cells.

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