THE IMMUNE RESPONSE AGAINST HAPTEN-AUTOLOGOUS PROTEIN CONJUGATES IN THE MOUSE

III. Specificity of Cooperating Non-Thymus-Processed (B) and Thymus-Processed (T) Lymphocytes*

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In order to achieve a deeper understanding of the regulation of the immune processes it is mandatory to further characterize the participants at the cellular and preferably also at the molecular level. Antibody formation frequently requires the collaboration between two types of lymphocytes where thymus-processed lymphocytes (T cells)[1] can function as helpers for bursa-type lymphocytes (non-thymus-processed lymphocytes or B cells) to become induced into high-rate antibody synthesis (1–4). In this scheme both groups of lymphocytes can be shown to carry specific immunocompetence. The way through which they express their potential competence can be shown to be linked to the presence on the outer membrane of these cells of specific receptors with specificity for the antigen (5–12). This demonstration has been relatively easy with regard to the B lymphocytes, but has encountered several difficulties when it comes to the T cells (13–16). As it has been so difficult to directly analyze the receptor for antigen on T cells by any direct measure, several attempts have been made to characterize the receptor(s) according to the capacity of various immunogens to selectively affect the function of normal or primed T cells (17–22). Comparisons have been made trying to establish whether T and B cells display receptors recognizing the very same spectrum of antigenic specificities, and the results have been quite contradictory (17–27).

The present experiments were performed to obtain further evidence on the speci-

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[1] *Abbreviations used in this paper:* B cells, non-thymus-processed lymphocytes; BGG, bovine gamma globulin; BSS, balanced salt solution, pH 6.9; ChGG, chicken gamma globulin; DNP, dinitrophenyl group; DNPF, 1-fluoro-2,4-dinitrobenzene; DNP-lys, DNP-lysine; HSA, human serum albumin; 2-ME, 2-mercaptoethanol; MIg, mouse gamma globulin; MSA, mouse serum albumin; NADs, new antigenic determinant; NMS, normal mouse serum; NRS, normal rabbit serum; OA, ovalbumin; PBS, 1:15 M phosphate-buffered saline, pH 7.4; RFC, rosette-forming cell; RRBC, rabbit erythrocytes; RSA, rabbit serum albumin; RtSA, rat serum albumin; SA, *Salmonella adelaide*; Skp, skin painted; SRBC, sheep erythrocytes; T cells, thymus-processed lymphocytes.

ficity of B and T cell receptors for antigen, either directly by filtration through antigen-coated columns (5, 6, 27) or by functional criteria. As immunogens we have chosen hapten-autologous protein conjugates as we believe that the immune reactivity against such immunogens is (*a*) more restricted with regard to the number of antigenic sites as compared with heterologous protein conjugates and thus more readily analyzed (28–30, footnotes 2 and 3) and (*b*) because such molecules can be considered as one of several model immunogens against which autoimmune or antitumor-specific reactions might have to exert their immune effects.

It has been shown previously that hapten-autologous protein conjugates contain two groups of antigenic sites, one hapten specific and the other one specific for new antigenic determinants (NADs), appearing in the hapten-protein conjugate as a consequence of the hapten coupling reaction (28, 29, footnote 2). The NADs behave as separate immunogenic and serologic determinants in animals that are either high or low responders to the haptenic group (29). Furthermore, the primary immune response to hapten-autologous albumin conjugates was found to be thymus dependent[4] (30), and the secondary response to these conjugates was found dependent on "conjugate"-specific helper cells (footnote 3).

In the present article we describe results obtained from studying the immune response in mice against mouse serum albumin (MSA) coupled to various degrees with the dinitrophenyl hapten (DNP). In such systems we have been able to demonstrate the existence of specific B cells with surface receptors either for NADs or DNP using specific fractionation on antigen-coated beads. The fine structure of the receptors for the NADs was thus analyzed.

Studies on the specificity of T cells reacting against the DNP-MSA conjugates were made possible by the use of special column filtration removing selectively all B cells (31, 32) followed by analysis of helper capacity of these cells in transfer systems. DNP-MSA-reactive T cells could be shown to be highly specific for DNP-MSA conjugates with only one exception: DNP-rat serum albumin (RtSA) is an immunogen showing cross-reactivity at the level of NADs with DNP-MSA (29), and a similar cross-reactivity was observed at the level of helper T cells. The implications of these results will be discussed.

Materials and Methods

Animals.—Inbred mice of the following genotypes were used throughout the experiments: AKR (*H-2^k*), CBA (*H-2^k*), CBA × ACA (*H-2^k/H-2^f*), CBA × BALB/c (*H-2^k/H-2^d*), and DBA × AKR (*H-2^d/H-2^k*). They were bred in this institute and in Statens Seruminstitut,

[2] Rubin, B., and B. Aasted. 1973. Characterization of new antigenic determinants introduced into autologous albumin by dinitrophenylation and sulfanalation. Manuscript submitted to *Immunology.*

[3] Rubin, B., V. Schirrmacher, and H. Wigzell. 1973. The immune response against hapten-autologous protein conjugates in the mouse. II. Carrier specificity in the secondary response and evidence for the existence of specific helper cells. *Scand. J. Immunol.* In press.

[4] Rubin, B., K. Hiesche, V. Schirrmacher, and G. Wigzell. 1973. The immune response against hapten-autologous protein conjugates in the mouse. IV. The thymus dependency of the primary response. Manuscript submitted to *J. Immunol.*

Copenhagen. All animals were between 2 and 6 mo of age at the beginning of the experiments. Recipient mice in the transfer experiments received 500 R whole body irradiation 2–6 h before cell transfer as described (footnote 3). Unless otherwise stated, mice were immunized with a standard hapten-carrier emulsion (in complete Freund's adjuvant) containing 1 mg protein/ml. Each mouse received a total of 0.2 ml of emulsion distributed subcutaneously (s.c.) in the hind footpads and two places at the abdomen.

Immunogens.—Mouse serum albumin (MSA), rat serum albumin (RtSA), and hapten-carrier conjugates were prepared as described previously (29). Heterologous proteins were obtained from Koch-Light Laboratories, Ltd. (Colnbrook, Buckinghamshire, England). The following DNP conjugates were used: $DNP_{12}BGG$, $DNP_{15}HSA$, $DNP_{6-8}MSA$, DNP_7OA, DNP_8RSA, and DNP_4RtSA (dinitrophenylated bovine gamma globulin, human serum albumin, mouse serum albumin, ovalbumin, rabbit serum albumin, and rat serum albumin, respectively). DNP-lysine (DNP-lys) was obtained from Mann Research Laboratories, Inc. (New York).

Antisera.—Rabbit polyvalent antimouse gamma globulin antiserum (anti-MIg) used for the rosette-inhibition studies was prepared as described (33). Rabbits were hyperimmunized with rabbit erythrocytes (RRBC) coated with mouse anti-RRBC antiserum. The anti-MIg antiserum was absorbed with sheep erythrocytes (SRBC) and passed through an MSA-Sepharose bead column to remove anti-MSA activity. The rabbit polyvalent antimouse gamma globulin antiserum used to make the anti-MIg-coated columns was prepared by immunizing rabbits with *Salmonella adelaide* (SA) bacteria coated with mouse anti-SA (31).

Anti-θ antiserum was used to determine the number of T lymphocytes in the different cell suspensions in the present experiments (34). The anti-θ antiserum was prepared by immunizing 2–3-mo old AKR female mice with 10^7 female CBA thymocytes at weekly intervals for 8 wk. The injections were performed intraperitoneally (i.p.) and s.c. alternately. The mice were bled 7 and 10 days after the last injection. The pooled antiserum was heat inactivated for 30 min at 56°C. In the presence of guinea pig complement (diluted 1:4) this antiserum was cytotoxic (100%) at a dilution of more than 1:27 against CBA and BALB/c thymocytes but not against AKR thymocytes. When used in the concentration of 0.1 ml (undiluted or diluted 1:3–1:9) per 10^7 lymphoid cells, 38–58% of CBA spleen cells and 55–75% of CBA lymph node cells were lysed by addition of complement. Further, the anti-θ antiserum destroyed the ability to express delayed hypersensitivity and the helper capacity but not the antibody-forming capacity of immune cells subsequently transferred and challenged in vivo.[5]

The cytotoxic test was performed as follows: Four tubes (nos. 1–4) received each 10^7 lymphoid cells. In two tubes (nos. 1–2) the cells were resuspended in 0.5 ml of 10% normal mouse serum in balanced salt solution (NMS/BSS), and in the other two tubes (nos. 3–4) the cells were resuspended in 0.5 ml of anti-θ antiserum. After 1 h at 4°C, the tubes were centrifuged, the supernatants removed, and the cells resuspended in 1 ml of 10% NMS/BSS (tubes 1 and 3) or fresh guinea pig serum diluted 1:4 in BSS (tubes 2 and 4). Incubation proceeded for 30 min at 37°C. Then, the number of trypan blue-excluding cells was determined in each tube. The results were expressed as percentage of live cells compared with the 10% NMS/BSS control. The percentage of θ-positive cells was calculated using the complement control as 100%:

$$100 - \frac{\text{anti-}\theta + C'}{C'} \times 100.$$

Cell Transfer.—The general design of the experiments was that introduced by Mitchison (35). Cells from mice immune against hapten-protein A conjugate were mixed in vitro with

[5] Rubin, B., and H. Wigzell. 1973. On the nature of hapten reactive helper lymphocytes. *Nat. New Biol.* In press.

cells from mice immune against protein B. This cell mixture was transferred to sublethally irradiated recipient mice that were stimulated with hapten-protein B conjugate, and the antihapten response was tested. The former cells will be called "B cells" and the latter cells will be called "T cells" throughout this paper, whether modulated by fractionation procedures to be described or not.

Cell donor mice were immunized as described above and lymphoid cells were harvested 10–40 days later in balanced salt solution (BSS). Spleen and lymph node cells (popliteal, inguinal, and axillary) were gently pressed through a stainless steel mesh and further homogenized by passage up and down through a pasteur pipette. The suspension was allowed to settle for a few minutes and the sediment discarded. The cells were washed three times in BBS. 20×10^6 trypan blue-excluding cells to be tested for B cell activity (antibody-forming precursor cell activity) and $2–20 \times 10^6$ trypan blue-excluding cells to be tested for T cell activity (helper cell activity) were injected intravenously (i.v.) together with antigen into the recipient mice. 10 days later the mice were bled and the individual antisera titrated for their antibody content.

Serology.—Antihapten antibodies were detected by passive hemagglutination using the microtiter system (Flow Laboratoiies Svenska AB, Solna, Sweden). DNP_7OA and $DNP_{15}HSA$ were coupled to SRBC using glutaraldehyde as coupling reagent (28, 29), and the titrations were performed in 1% normal rabbit serum in phosphate-buffered saline (NRS in PBS). The DNP_7OA-SRBC were used to detect anti-DNP antibodies except in experiments where OA was used as a carrier. In the latter case, anti-DNP antibodies were measured against DNP_{15}-HSA-SRBC in presence of 10^{-5} M HSA. The hemagglutination assay using DNP_7OA- and $DNP_{15}HSA$-SRBC was of similar sensitivity, and the anti-DNP titers could be inhibited completely by DNP-lys (10^{-3}–10^{-5} M).

Antibodies to the NADs were measured by hemagglutination against DNP_7MSA-SRBC (glutaraldehyde complex). The titrations were performed as above but in the presence of 10^{-3} M DNP-lys. This DNP-lys concentration inhibited any anti-DNP titer (29). Thus the resulting titer gave a quantitative measurement of the antibody content directed against the NADs (for further details see references 28, 29). This anti-NAD titer could be inhibited only by $DNP_{6-8}MSA$ (10^{-4}–10^{-7} M).

Anticarrier antibodies were detected by hemagglutination against carrier-coated SRBC (28). The results are expressed as geometric means of sera from at least five mice per group. The amount of 7S hemagglutinin in the antisera were determined by the 2-mercaptoethanol (2-ME) method (see reference 28). 2-ME-resistant antibodies will be called 7S antibodies.

Rosette Assay.—The amount of anti-DNP B cells in the cell suspensions to be transferred into recipient mice and in column-passed cell suspensions was tested by the rosette-forming cell (RFC) assay (34, 37). Target cells were SRBC coated with $DNP_{12}ChGG$ (dinitrophenylated chicken gamma globulin with anti-SRBC activity). ChGG was prepared by hyperimmunization of chickens with SRBC. After ammonium sulfate precipitation and passage through Sephadex G-200, the ChGG was conjugated with DNP as described (29). The DNP_{12}-ChGG conjugates had a $\log_2$ anti-SRBC titer of 8.0. It was coupled to SRBC as follows: 50 μl of $DNP_{12}ChGG$ + 10 ml of 2% SRBC in PBS was incubated for 30 min at 37°C and then washed three times in PBS.

5×10^6 lymphoid cells (in 0.1 ml) were mixed at 4°C with 0.1 ml of the 2% $DNP_{12}ChGG$-SRBC (i.e. lymphoid cell to target cell ratio = 1:8). The mixture was immediately centrifuged for 10 min at 200 *g* (4°C), cold PBS added up to 2.0 ml, and the pellet was resuspended very carefully with a pasteur pipette. The number of RFCs was counted in a hemacytometer at room temperature. An RFC was defined as a "lymphocytic" cell having five or more target erythrocytes bound to its surface. Macrophage rosettes were excluded only by morphology. A minimum number of 30 RFCs in immune cell suspensions and 10 RFCs in normal and anti-MIg column-passed cell suspensions was counted. The results were expressed as number

of RFCs per 10^6 viable lymphocytes, i.e., anti-$\log_{10}$ to the geometric mean of six to eight determinations per suspension.

When hapten-inhibition experiments were carried out, 0.1 ml of buffer or 0.1 ml of DNP-lys (10^{-4}–10^{-9} M) was added to the lymphoid cell suspension and the mixture incubated for 30 min at 4°C before addition of target cells. The number of RFCs in a cell suspension treated with anti-θ antiserum plus complement was determined after readjustment of the number of viable cells to 5×10^6/0.1 ml. The inhibitory capacity of rabbit anti-MIg antiserum on rosette formation was tested by incubating 5×10^6 lymphoid cells (in 0.1 ml) with 0.1 ml of the antiserum diluted 1:20 for 30 min at 4°C before addition of target cells. Inhibition with rabbit anti-MIg antiserum could be abolished completely by suspending the lymphoid cells in 10^{-5} M MIg.

RFCs measured as described above were of B cell type based on the following observations (Table I): (*a*) They could be inhibited completely by rabbit anti-MIg antiserum (38, 39). (b) They were not sensitive to anti-θ antiserum plus complement. (*c*) They were inhibited 50% by DNP-lys in relative low concentrations (10^{-6}–10^{-9} M) (24). (*d*) They displayed the expected increase in affinity with time after immunization (40, 41). As 10^{-4} M DNP-lys inhibit almost completely the DNP-RFCs (Table I), this concentration of DNP-lys was used in the column separation experiments to be described.

Antigen-Specific Columns.—DNP coupled to MSA or OA was chemically linked to Sepharose 6B supersize (800–1500 μm) beads (kindly donated by Pharmacia, Uppsala, Sweden) by means of CNBr (27). 15 g of Sepharose was suspended in 25 ml of distilled water, the pH

TABLE I

RFC against DNP-ChGG-SRBC Per 10^6 Lymphoid Cells

Cells*	Inhibitor	DNP-RFC ± SD‖		SRBC-RFC ± SD	
		Expt. 1	Expt. 2	Expt. 1	Expt. 2
I.C.	–	3,920 ± 290	5,780 ± 475	333 ± 95	480 ± 215
"	10^{-8} M DNP-lys	3,210 ± 510	2,570 ± 390	–	–
"	10^{-6} M "	1,735 ± 470	1,039 ± 195	–	–
"	10^{-4} M "	890 ± 310	400 ± 205	310 ± 103	495 ± 193
"	Anti-θ + C'‡	6,750 ± 615	11,800 ± 335	573 ± 201	–
"	Anti-MIg§	80 ± 120	105 ± 195	35 ± 83	75 ± 100
"	Anti-MIg + 10^{-5} M MIg	3,760 ± 570	5,890 ± 435	290 ± 109	501 ± 187
N.C.	–	680 ± 290	515 ± 195	450 ± 87	575 ± 203
"	10^{-4} M DNP-lys	667 ± 113	575 ± 310	397 ± 119	590 ± 190
"	Anti-θ + C'	1,170 ± 178	981 ± 290	385 ± 205	–
"	Anti-MIg	75 ± 105	30 ± 95	–	85 ± 105

* I.C. = immune cells from CBA × BALB/c mice immunized with 200 μg of DNP_8MSA 14 days before the test (expt. 1) or 35 days before the test (expt. 2). N.C. = normal cells from CBA × BALB/c mice.

‡ Treatment with anti-θ-plus complement, see Materials and Methods.

§ 0.1 ml of rabbit anti-MIg antiserum diluted 1:10 incubated with 0.1 ml of lymphoid cells (10^7 cells) for 30 min at 4°C in the presence or absence of 10^{-5} M MIg.

‖ DNP-ChGG-SRBC-specific RFC; anti-$\log_{10}$ to the geometric mean of six determinations per suspension, the SRBC-RFC not subtracted.

adjusted to 11.0, and 2 g of CNBr was added. The pH was kept at 11 with 2 N NaOH during the reaction (about 30 min). Thereafter, the activated Sepharose was washed with 2 liters of ice-cold water. Then, 10 ml of 0.2 M bicarbonate buffer pH 9.0 was added, the pH checked, and 200 mg of DNP_7MSA or DNP_7OA in 20 ml of bicarbonate buffer added. The pH was adjusted to 9.0 and the coupling reaction continued for 2 days with stirring in the cold. Then the coupled beads were placed in glass columns (1.5 × 30 cm [Pharmacia]), the supernatant was collected for protein determination, and the beads were washed with bicarbonate buffer, pH 9.0, until OD 280 was below 0.010. The beads were then incubated overnight in the cold with 50 ml of a 0.05 M ethanolamine bicarbonate solution. After extensive washing with PBS and the beads again placed in a glass column, they were finally incubated with 5 ml of normal rabbit serum.

The amount of DNP conjugate bound to the Sepharose was determined by measuring the protein content of the supernatants from the coupling reaction. The biuret method was used. 155 mg of DNP_7OA and 180 mg of DNP_7MSA was bound to the Sepharose beads, respectively.

10 g of DNP-conjugated Sepharose beads placed in a glass column (1.5 × 30 cm) was added 10 ml of a 2% NRS/BSS solution. To this was added 20 ml of the cell suspension to be absorbed (5×10^7 cells/ml). The beads and the cells were mixed by gentle agitation, and the column was placed in a horizontal position at room temperature and left for 1 h before elution of the cells. Cell yield by this procedure was between 50–60% of the input. After cell separation the beads were washed first with 0.1 M acetic acid, then with 0.2 M bicarbonate buffer, pH 10.0, and finally with PBS. This procedure allowed us to use the same bead material for more than 20 experiments.

Antimouse Immunoglobulin-Coated Columns.—Anti-MIg-coated columns were prepared as described previously (31, 32, footnote 3). Degalan V26 beads (Degussa Wolfgang AG, Hanau am Main, Germany) were coated with a 0.5% MIg solution in PBS. The beads were subsequently poured into a glass column (1.5 × 90 cm [Pharmacia]) and washed extensively with PBS. The column was then filled with rabbit anti-MIg diluted 1:2–5, left for 2 h at 4°C, and finally washed again with PBS. The amount of anti-MIg put onto the column was such that the $\log_2$ hemagglutination titer against MIg-SRBC was reduced less than one $\log_2$ step after application on the column. A suspension of mouse immune cells (5×10^8–20×10^8 cells at a concentration of 2×10^7 cells per ml) was passed through the column with a flow rate of 2 ml/min. Cell yield was approximately 20% of the input.

The passed cells have been shown to contain: (*a*) membrane immunoglobulin-negative cells (31), (*b*) helper cells (42, the present paper, and footnote 3), (*c*) cells mediating specific in vitro cytotoxicity against allogeneic target cells (32), (*d*) cells responding by DNA synthesis to soluble antigen in vitro,[6] (*e*) anti-θ-positive cells (the present paper) and cells sensitive to a rabbit antimouse thymocyte-specific antiserum (31, 32). The retained cells contained: (*a*) membrane immunoglobulin-positive cells (31), (*b*) plaque-forming cells (42), (*c*) RFC (the present paper), (*d*) antibody-forming cell precursors (31, 42, footnote 3), (*e*) cells mediating specific in vitro cytotoxicity against soluble antigens complexed to chicken red cells.[7] Thus, by these criteria, the passed cells can be classified as T lymphocytes. The B lymphocytes are retained on the columns.

Statistics.—Statistical significance of the hemagglutination results was evaluated by the Student's *t* test. A difference of two $\log_2$ units or more between the experimental groups was found significant ($P \leq 0.01$). Standard deviations (SD) of the means, usually not given in the tables, ranged from 0.40 to 1.30.

[6] Rubin, B., and H. Wigzell. 1973. Manuscript in preparation.

[7] Golstein, P., V. Schirrmacher, B. Rubin, and H. Wigzell. 1973. Cytotoxic immune cells with specificity for defined soluble antigen. II. Chasing the killing cells. *Cell. Immunol.* In press.

Statistical significance of the RFC counts was likewise evaluated by the Student's t test. Geometric means and standard deviations were calculated on basis of at least six determinations.

RESULTS

In Vitro Investigation of Nonpassed and Column-Passed Cells.—Control cell suspension and column-passed cell suspensions to be used in the subsequent transfer experiments were tested in vitro for (*a*) the ability to form DNP-specific RFCs and (*b*) the content of θ-positive cells (T cells [34]).

Table II shows that both the DNP_7OA- and the DNP_7MSA-Sepharose columns have depleted completely the passed cell suspension for DNP-specific RFCs without affecting the number of SRBC-specific RFCs. Table III shows

TABLE II

RFC against $DNP_{12}ChGG$-SRBC Per 10^6 Lymphoid Cells

Cells*	Column	Inhibitor	DNP-RFC‡		SRBC-RFC	
			Expt. 1	Expt. 2	Expt. 1	Expt. 2
DNP_6MSA	–	–	3,550	1,925	805	475
"	–	10^{-4} M DNP-lys	680	438	840	443
"	DNP_7OA-Sepharose	–	795	–	837	–
"	"	10^{-4} M DNP-lys	705	–	825	–
"	DNP_7MSA-Sepharose	–	–	555	–	513
"	"	10^{-4} M DNP-lys	–	517	–	535

* DNP_6MSA immune cells from: expt. 1 = expt. 2 in Table V; expt. 2 = 4-wk old immune cells passed through a DNP_7MSA-Sepharose column.

‡ $DNP_{12}ChGG$-SRBC-specific RFC; anti-$\log_{10}$ to the geometric mean of six determinations per suspension, the SRBC-RFC not subtracted.

TABLE III

RFC against $DNP_{12}ChGG$-SRBC Per 10^6 Lymphoid Cells

Cells*	Column	Inhibitor	DNP-RFC§			SRBC-RFC		
			Expt. 1	Expt. 2	Expt. 3	Expt. 1	Expt. 2	Expt. 3
DNP-MSA	–	–	3,735	2,985	4,150	605	480	585
"	–	10^4 M DNP-lys	690	525	605	635	463	601
"	–	Anti-θ + C'‡	6,969	6,757	8,479	1,090	965	1,148
"	Anti-MIg	–	85	35	103	73	35	95
"	"	10^{-4} M DNP-lys	85	40	85	67	38	87

* DNP_6MSA immune cells: expt. 1 = expt. 1 in Table VII; expt. 2 = expt. 2 in Table VII; expt. 3 = $DNP_{32}MSA$ immune cells.

‡ See Table I.

§ $DNP_{12}ChGG$-SRBC-specific RFC calculated as in Table I: SRBC-RFC not subtracted.

two things. (*a*) Anti-θ antiserum plus complement does not inhibit the formation of DNP-specific RFCs. In fact, no θ-positive T cells making rosettes with DNP-SRBC were ever found, i.e., the number of DNP-specific RFCs in DNP-MSA immune cell suspensions was always increased after treatment with anti-θ antiserum plus complement, and the increase was equivalent to the decrease in the number of living cells after treatment. (*b*) The anti-MIg-coated column depleted drastically the number of RFCs against both DNP and SRBC. The number of DNP- and SRBC-RFCs in anti-MIg column-passed cell suspensions was similar to the number of RFCs found in cell suspensions inhibited with rabbit anti-MIg before incubation with the DNP-ChGG-SRBC target cells (compare Table III with Table I).

Table IV gives a summary of representative experiments where control and column-passed cell suspensions were tested for the content of θ-positive cells. The antigen-coated columns did not change the number of θ-positive cells, whereas the anti-MIg column-passed cells were highly enriched in θ-positive cells. These findings are in agreement with previously published experiments where the enrichment of T cells in anti-MIg column-passed cells were tested with a rabbit antimouse thymocyte-specific antiserum (31, 32). However, the latter antiserum killed in most cases 5–10% more of the anti-MIg column-passed cells than did the anti-θ antiserum. This might be due to the relatively low concentration of the θ-antigen on some T lymphocytes and that the rabbit

TABLE IV

Effect of Anti-θ Antiserum on Control and Column-Passed Cells

Expt. no.*	Column	Live cells in cytotoxic test‡		θ-positive§ cells
		C′	Anti-θ + C′	
		%	%	%
1	–	88	40	55
1	DNP_7OA-Sepharose	87	38	57
2	–	85	50	41
2	DNP_7MSA-Sepharose	89	51	43
3	–	91	46	49
3	MIg	89	13	85
4	–	85	48	53
4	MIg	90	5	94
5	–	83	42	50
5	MIg	92	12	87

* Expt. 1 = expt. 2 in Table V; expt. 2 = expt. 1 in Table VI; expt. 3 = expt. 1 in Table VII; expt. 4 = expt. 2 in Table VII; expt. 5 = expt. in Table VIII.

‡ Calculated as described in Materials and Methods. C′ = guinea pig complement diluted 1:4.

§ Percent θ-positive cells = $100 - \frac{\text{anti-}\theta + C'}{C'} \times 100$.

antithymocyte antiserum is directed against membrane antigens other than the θ-antigen.

Specific Depletion of B Lymphocytes from DNP-MSA Immune Cell Suspensions.—We have shown previously (29) that antibodies produced against $DNP_{6-8}MSA$ could be separated into two kinds: (*a*) those specific for the DNP group and (*b*) those specific for the NAD groups. The latter antibodies had no detectable affinity for the hapten. We asked the question whether B cells induced by DNP_6MSA could be separated into two major groups; those specific for DNP and NAD, respectively.

There seems to be no cross-reactivity at the anti-NAD antibody level between DNP_6MSA and DNP_7OA (29, 30, footnote 3). Therefore, DNP_7OA was coupled to Sepharose as DNP-specific immunoabsorbent, and DNP_7MSA was coupled to Sepharose as DNP + NAD-specific immunoabsorbent. Further, as 10^{-4} M DNP-lys inhibits completely the ability of DNP_6MSA immune cells to form rosettes against $DNP_{12}ChGG$-SRBC (Table I), one would predict (in analogy with the antibody situation) that it should be possible to absorb anti-NAD-specific B cells on DNP_7MSA-Sepharose if the separation was carried out in the presence of 10^{-4} M DNP-lys.

As can be seen in Table V the two experiments clearly show a specific depletion of anti-DNP-reactive memory cells of the B cell type. The DNP_7OA column-passed DNP_6MSA immune cells gave no cooperative responses when mixed with OA helper cells and stimulated with DNP_7OA (group 7 compared with groups 3 and 10), and they gave a greatly reduced homologous response

TABLE V

Specific Depletion of DNP-Reactive B Lymphocytes on DNP_7OA-Sepharose Bead Columns

Group no.	Cells transferred		Column	Antigen§	Log₂ hemagglutination‖ titer			
					Expt. 1		Expt. 2	
	B cells*	T cells‡			Anti-DNP	Anti-NAD	Anti-DNP	Anti-NAD
1	2×10^7 DNP_6MSA-C	–	–	DNP_6MSA	6.5	8.3	5.6	6.9
2	5×10^6 "	–	–	"	4.1	6.0	–	–
3	2×10^7 "	2×10^7 OA	–	DNP_7OA	–	–	4.8	0.3
4	2×10^7 DNP_6MSA-P	–	DNP_7OA-Sepharose	DNP_6MSA	2.3	7.9	1.6	6.4
5	5×10^6 "	–	"	"	1.0	5.8	–	–
6	2×10^7 "	–	"	DNP_7OA	0.7	0.0	–	–
7	2×10^7 "	2×10^7 OA	"	"	0.3	0.0	0.9	–
8	2×10^7 N.C.	–	–	DNP_6MSA	0.9	0.3	–	–
9	2×10^7 "	–	–	DNP_7OA	1.7	0.0	–	–
10	2×10^7 "	2×10^7 OA	–	"	2.6	0.0	2.3	0.0

* B cells were mixed spleen and lymph node cells from CBA × ACA mice immunized 6 wk before transfer (expt. 1) or from CBA × BALB/c mice immunized 4 wk before transfer (expt. 2). C = control cells; P = passed cells.

‡ T cells were mixed spleen and lymph node cells from syngeneic mice immunized with OA 6 wk before transfer.

§ 10 μg of antigen given together with the cells.

‖ Anti-DNP = $\log_2$ hemagglutination titer against $DNP_{15}HSA$-SRBC. Anti-NAD = $\log_2$ hemagglutination titer against DNP_7MSA-SRBC in the presence of 10^{-3} M DNP-lys. All titers were 7S antibody titers and the figures are geometric means of six mice per group.

(groups 4 and 5 compared with groups 1 and 2). The anti-NAD response of the DNP_7OA column-passed DNP_6MSA immune cells was not affected (groups 4 and 5 vs. groups 1 and 2). This means (*a*) that DNP-reactive memory cells of the B cell type could be specifically separated from the NAD-reactive memory cells and (*b*) that DNP_6MSA-specific helper cells were not absorbed (the latter are necessary for the induction of the secondary anti-NAD response).

Table VI represents two experiments showing that anti-NAD memory cells could be separated from anti-DNP memory cells, when the absorption was carried out on DNP_7MSA-Sepharose beads in the presence of 10^{-4} M DNP-lys.

TABLE VI

Specific Depletion of NAD-Reactive B Lymphocytes on DNP-MSA-Sepharose Bead Columns

Group no.	Cells transferred		Column	Antigen§	Log₂ hemagglutination titer‖			
					Expt. 1		Expt. 2	
	B cells*	T cells‡			Anti-DNP	Anti-NAD	Anti-DNP	Anti-NAD
1	2×10^7 DNP_6MSA-C	–	–	DNP_6MSA	5.9	9.1	5.2	4.7
2	5×10^6 "	–	–	"	4.2	7.8	–	–
3	2×10^7 "	2×10^7 OA	–	DNP_7OA	–	–	5.5	0.3
4	2×10^7 DNP_6MSA-P	–	DNP_7MSA-Sepharose + DNP-lys 10^{-4} M	DNP_6MSA	5.7	0.9	5.4	1.2
5	5×10^6 "	–	" "	"	3.8	0.0	–	–
6	2×10^7 "	–	" "	DNP_7OA	0.9	0.0	1.2	0.4
7	2×10^7 "	2×10^7 OA	" "	"	5.1	0.0	6.6	0.6

* B cells were mixed spleen and lymph node cells from CBA × ACA mice immunized 4 wk before transfer (expt. 1) or from AKR mice immunized 3 wk before transfer (expt. 2). C = control cells; P = passed cells. Both C and P cells were incubated with DNP-lys (10^{-4} M) and washed once before transfer.

‡ T cells were mixed spleen and lymph node cells from syngeneic mice immunized with OA 6 wk before transfer.

§ 10 μg of antigen given together with the cells.

‖ See Table V.

Again, DNP_6MSA-specific helper cells were not absorbed, i.e., no difference in the anti-DNP response of nonpassed and passed cells.

Specificity of Helper Cells Induced by DNP-MSA Conjugates.—$DNP_{12}BGG$ and DNP_6MSA do not cross-react at the NAD level (29, 30, footnote 3). As shown in Table VII $DNP_{12}BGG$ immune cells do not respond to DNP_6MSA as secondary stimulus. When adding different numbers of anti-MIg column-passed DNP_6MSA immune cells, which give no anti-DNP response by themselves (groups 6 and 11), good cooperative anti-DNP responses were obtained with cell numbers of 10^7 or more. When using DNP_7OA as secondary antigen in the same cellular combination, no anti-DNP response was obtained (group 8 vs. 7).

Some experiments were performed to test whether differences were obtained

TABLE VII

Helper Effect of DNP_6MSA-Specific T Cells

Group no.	Cells transferred		Column	Antigen§	Log_2 anti-DNP titer‖	
	B cells*	T cells‡			Expt. 1	Expt. 2
1	2×10^7 $DNP_{12}BGG$	–	–	$DNP_{12}BGG$	8.9	8.6
2	"	–	–	DNP_6MSA	0.7	0.0
3	"	30×10^6 DNP_6MSA-P	Anti-MIg	"	–	7.3
4	"	10×10^6 "	"	"	6.7	4.6
5	"	3×10^6 "	"	"	1.8	1.6
6	–	20×10^6 "	"	"	1.1	0.2
7	2×10^7 $DNP_{12}BGG$	–	–	DNP_7OA	3.3	2.7
8	"	10×10^6 DNP_6MSA-P	Anti-MIg	"	3.7	2.9
9	"	–	–	–	0.9	0.3

* B cells were mixed spleen and lymph node cells from CBA × ACA mice (expt. 1) or CBA × BALB/c mice (expt. 2) immunized 6–8 wk before transfer.

‡ T cells were mixed spleen and lymph node cells from syngeneic mice immunized 3 wk before transfer. The cells were passed through an anti-MIg-coated column before transfer.

§ 10 μg of antigen given together with the cells.

‖ Log_2 hemagglutination titer against $DNP_{15}HSA$-SRBC. All titers were 7S titers and the results were geometric means of five mice per group.

when using antigen column-passed or anti-MIg column-passed cells as helper cells. In the former cell suspension only anti-DNP-specific B cells were retained, in the latter cell suspension virtually all B cells were retained (Table IV). Table VIII shows the results from a representative experiment where DNP_6-MSA immune cells were tested after absorption on DNP_7OA- or DNP_7MSA-coated columns. Different numbers of nonspecific B cells (groups 3–8) or T cells (groups 9–11) had no impact on the anti-DNP response obtained. The anti-MIg column-passed cells usually gave a better "helper function" per cell number than did antigen column-passed cells, a finding expected already from the degree of T cell enrichment by the two kind of columns.

The depletion of DNP-reactive memory cells was always most significantly seen when tested in a cooperative response. This might be due to (*a*) the sensitivity of the homologous stimulation vs. the cooperative stimulation and/or (*b*) the stimulation in the homologous situation of anti-DNP cells of low avidity for DNP or anti-"DNP" cells with specificity for the DNP-NAD complex. The latter possibility is supported by the findings in Table VIII which shows (*a*) that the DNP_7MSA-Sepharose column depleted the DNP_6MSA immune cell suspension for both anti-DNP B cells and anti-NAD B cells and (*b*) that anti-DNP B cells were retained more efficiently with the DNP_7MSA-Sepharose beads than with the DNP_7OA beads (compare groups 5, 8, and 12).

Cross-Reactivity of DNP-MSA-Specific T Cells.—As shown in Table VII (see also reference 30 and footnote 3) DNP-MSA-specific T cells did not react with DNP_7OA. When DNP_8HSA, DNP_8RSA, or DNP_7OA immune cells were passed through an anti-MIg-coated column and mixed with $DNP_{12}BGG$

TABLE VIII

Helper Capacity of DNP_6MSA Cells Passed through Antigen or Anti-MIg-Coated Columns

Group no.	Cells transferred		Column	Antigen§	Log_2 hemagglutination titer‖	
	B cells*	T cells‡			Anti-DNP	Anti-NAD
1	2×10^7 $DNP_{12}BGG$	–	–	$DNP_{12}BGG$	9.8 ± 0.51	0.0
2	"	–	–	DNP_6MSA	1.1 ± 0.93	0.0
3	"	20×10^6 DNP_6MSA-P	DNP_7OA-Sepharose	"	5.1 ± 0.68	7.9 ± 0.83
4	"	5×10^6 "	" "	"	1.9 ± 0.77	5.1 ± 0.71
5	–	20×10^6 "	" "	"	2.3 ± 0.59	8.1 ± 0.93
6	2×10^7 $DNP_{12}BGG$	20×10^6 "	DNP_7MSA-Sepharose	"	5.3 ± 0.73	0.0
7	"	5×10^6 "	" "	"	1.7 ± 0.87	0.0
8	–	20×10^6 "	" "	"	0.9 ± 0.59	1.1 ± 0.91
9	2×10^7 $DNP_{12}BGG$	20×10^6 "	Anti-MIg	"	7.3 ± 0.85	0.0
10	"	5×10^6 "	"	"	3.8 ± 0.99	0.0
11	–	20×10^6 "	"	"	0.6 ± 0.73	1.1 ± 0.96
12	2×10^7 DNP_6MSA	–	–	"	5.2 ± 0.68	7.4 ± 0.87
13	2×10^7 $DNP_{12}BGG$	–	–	–	0.7 ± 0.79	0.0

* B cells were mixed spleen and lymph node cells from CBA × BALB/c mice immunized 7 wk before transfer.

‡ T cells were mixed spleen and lymph node cells from CBA × BALB/c mice immunized 4 wk before transfer. The cells were passed through an antigen- or anti-MIg-coated column. (Percent θ-positive cells were: C = 49%; DNP_7OA-Sepharose = 51%; DNP_7MSA-Sepharose = 52%; anti-MIg-P = 89%.)

§ 10 μg of antigen given together with the cells.

‖ Anti-DNP = $\log_2$ hemagglutination titer against $DNP_{15}HSA$-SRBC. Anti-NAD = $\log_2$ hemagglutination titer against DNP_7MSA-SRBC in the presence of 10^{-3} M DNP-lys. All titers were 7S titers, and the results were geometric means of six mice per group (± one standard deviation).

immune cells, they were not stimulated with DNP-MSA conjugates (Table IX). To test whether T cells with DNP specificity were at all responsible for the helping capacity studied in the present system, we used cells from mice skin painted with 1-fluoro-2,4-dinitrobenzene (DNPF) (23, 24). These mice gave good delayed-type hypersensitivity when tested by the ear-swelling technique (43). Despite this, lymph node cells from these mice did not help the anti-DNP response of $DNP_{12}BGG$ immune cells restimulated with DNP_6MSA or DNP_7OA (Table X). In summary, these experiments seem to exclude the possibility of cooperation between DNP epitopes in the present situation (23).

We showed previously that only NADs introduced by DNP into RtSA showed cross-reactivity with the NADs in DNP-MSA (29). Therefore, DNP_8-MSA or DNP_4RtSA immune cells passed through an anti-MIg-coated column were tested for helper activity against DNP_4RtSA and DNP_8MSA, respectively. As can be seen in Table XI from 2×10^6 to 20×10^6 DNP_8MSA-specific T cells recognized determinants on the DNP_4RtSA molecule, in accordance with the cross-specificity of anti-NAD/MSA antibodies. DNP_4RtSA-specific T cells were tested in a similar cellular combination, and they also recognized determinants on the DNP_8MSA molecule (Table XII). However, there seemed to be quantitative differences between the number of cross-reactive cells in the

TABLE IX

Helper Capacity of DNP-Heterologous Protein Conjugate-Specific, Anti-MIg Column-Purified T Cells

Group no.	B cells*	T cells‡	Column	Antigen§	Log_2 anti-DNP titer‖
1	2×10^7 $DNP_{12}BGG$	-	-	$DNP_{12}BGG$	8.3 ± 0.91
2	"	-	-	DNP_6MSA	0.7 ± 0.39
3	"	-	-	DNP_8HSA	3.6 ± 0.63
4	"	2×10^7 DNP_8HSA	Anti-MIg	"	9.1 ± 0.87
5	"	"	"	DNP_6MSA	0.9 ± 0.47
6	"	2×10^7 DNP_8RSA	"	DNP_8RSA	10.2 ± 0.93
7	"	"	"	DNP_6MSA	1.1 ± 0.56
8	"	-	-	DNP_8RSA	3.7 ± 0.81
9	"	2×10^7 DNP_7OA	Anti-MIg	DNP_7OA	10.8 ± 0.97
10	"	"	"	DNP_6MSA	0.8 ± 0.51
11	"	-	-	DNP_7OA	1.9 ± 0.73
12	-	2×10^7 DNP_8HSA	Anti-MIg	DNP_8HSA	1.1 ± 0.72
13	-	" DNP_8RSA	"	DNP_8RSA	1.3 ± 0.69
14	-	" DNP_7OA	"	DNP_7OA	1.6 ± 0.75

* B cells were spleen and lymph node cells from DBA $\times$ AKR mice immunized with $DNP_{12}BGG$ 8 wk before transfer.

‡ T cells were spleen and lymph node cells from DBA $\times$ AKR mice immunized with DNP_8HSA, DNP_8RSA, or DNP_7OA 3 wk before transfer.

§ 10 μg of antigen were given together with the cells.

‖ Log_2 hemagglutination titer against $DNP_{12}ChGG$-SRBC. All titers were 7S titers and the results are geometric means of five per group.

TABLE X

Helper Effect of Lymph Node Cells from DNP-Skin-Painted Mice

Group no.	B cells*	T cells‡	Antigen§	Log_2 anti-DNP titer $\pm$ SD‖
1	2×10^7 $DNP_{12}BGG$	-	$DNP_{12}BGG$	6.8 ± 0.44
2	"	-	DNP_8MSA	0.8 ± 0.63
3	"	20×10^6 DNP-Skp	"	0.4 ± 0.36
4	"	10×10^6 "	"	0.7 ± 0.53
5	"	2×10^6 "	"	0.2 ± 0.33
6	-	20×10^6 "	"	0.0
7	2×10^7 $DNP_{12}BGG$	-	DNP_7OA	1.9 ± 0.57
8	"	20×10^6 DNP-Skp	"	2.1 ± 0.91
9	-	"	"	0.0

* B cells were mixed spleen and lymph node cells from DBA $\times$ AKR mice immunized 8 wk before transfer.

‡ T cells were lymph node cells from DBA $\times$ AKR mice skin painted with 100 μg of DNPF 7 days before transfer. Ear-swelling of 8 painted mice: 13.0 ± 1.7; ear-swelling of 10 normal mice: 2.0 ± 1.0 (U of 10^{-3} cm [43]).

§ 10 μg of antigen given together with the cells.

‖ Log_2 hemagglutination titers against $DNP_{15}HSA$-SRBC all titers are 7S antibody titers and results are geometric means of five mice per group.

DNP_8MSA-specific T cell population and in the DNP_4RtSA-specific T cell population, the former having more cells recognizing DNP_4RtSA than the latter recognizing DNP_8MSA (compare groups 5–9 in Tables XI and XII).

TABLE XI

Helper Effect of DNP_8MSA-Specific T Lymphocytes

Group no.	Cells transferred		Column	Antigen§	Log₂ hemagglutination titer‖			
					Expt. 1		Expt. 2	
	B cells*	T cells‡			Anti-DNP	Anti-NAD	Anti-DNP	Anti-NAD
1	2×10^7 DNP_8MSA	–	–	–	0.3	0.0	0.1	0.0
2	"	–	–	DNP_8MSA	4.8	7.2	7.1	6.7
3	"	–	–	DNP_4RtSA	5.1	3.8	7.0	4.2
4	"	–	–	$DNP_{12}BGG$	1.1	0.0	0.7	0.0
5	2×10^7 $DNP_{12}BGG$	–	–	DNP_4RtSA	0.8	0.0	0.9	0.0
6	"	20×10^6 DNP_8MSA-P	Anti-MIg	"	6.1	0.0	7.9	0.0
7	"	10×10^6 "	"	"	5.6	0.0	5.8	0.0
8	"	2×10^6 "	"	"	4.0	0.0	3.7	0.0
9	–	20×10^6 "	"	"	0.7	0.0	1.1	0.0
10	2×10^7 $DNP_{12}BGG$	–	–	DNP_8MSA	0.9	0.0	0.6	0.0
11	"	10×10^6 DNP_8MSA-P	Anti-MIg	"	5.1	0.0	5.9	0.0
12	–	"	"	"	1.4	0.8	1.2	1.0

* B cells were mixed spleen and lymph node cells from DBA × AKR mice immunized with $DNP_{12}BGG$ 10 wk before transfer, or immunized with DNP_8MSA 3 wk (expt. 1) or 6 wk (expt. 2) before transfer.

‡ T cells were mixed spleen and lymph node cells from DBA × AKR mice immunized with DNP_8MSA, see B cells. P = the cells passed through an anti-MIg column.

§ 10 μg of antigen given together with the cells.

‖ Anti-DNP = $\log_2$ hemagglutination titer against DNP_7OA-SRBC. Anti-NAD = $\log_2$ hemagglutination titer against DNP_7MSA-SRBC in the presence of 10^{-3} M DNP-lys. All titers are 7S antibody titers, and results are geometric means of five mice per group.

TABLE XII

Helper Effect of DNP_4RtSA-Specific T Lymphocytes

Group no.	Cells transferred		Column	Antigen§	Log₂ hemagglutination titer‖			
					Expt. 1		Expt. 2	
	B cells*	T cells‡			Anti-DNP	Anti-NAD	Anti-DNP	Anti-NAD
1	2×10^7 DNP_4RtSA	–	–	–	0.2	0.0	0.2	0.0
2	"	–	–	DNP_4RtSA	7.4	5.9	5.8	1.7
3	"	–	–	DNP_8MSA	3.2	5.6	1.9	1.2
4	"	–	–	$DNP_{12}BGG$	1.2	0.0	0.7	0.0
5	2×10^7 $DNP_{12}BGG$	–	–	DNP_8MSA	0.9	0.0	0.5	0.0
6	"	20×10^6 DNP_4RtSA	Anti-MIg	"	4.3	0.0	3.6	0.0
7	"	10×10^6 "	"	"	3.8	0.0	1.9	0.0
8	"	2×10^6 "	"	"	1.4	0.0	0.7	0.0
9	–	20×10^6 "	"	"	0.7	0.0	0.8	0.0
10	2×10^7 $DNP_{12}BGG$	–	–	DNP_4RtSA	1.1	0.0	1.4	0.0
11	"	10×10^6 DNP_4RtSA	Anti-MIg	"	8.0	0.0	6.9	0.0
12	"	20×10^6 "	"	"	1.9	0.0	1.6	0.0

* See Table XI.

‡ " " ".

§ " " ".

‖ " " ".

DISCUSSION

Specificity characteristics of B and T lymphocytes immune against hapten-autologous serum albumin conjugates in the mouse have been studied in the present article. Hapten-binding lymphocytes were made directly visible by the rosette cell technique (36, 37), and in the present system (DNP) such rosette-forming cells could be shown to be B lymphocytes according to functional and morphological criteria (Tables I, II, and III). The failure to find thymus-processed lymphocytes as rosette-forming cells is not surprising, in view of earlier reports on problems as to demonstrate significant uptake of antigen on T lymphocytes (44–46). In agreement with this, when studying the antigen-binding receptors on the immune lymphoid cells by attempts to selectively remove immunocompetent cells by incubation with antigen-coated beads in the present system, we were only able to remove specific B and not T cells by such maneuvers. Even if the incubation conditions were similar to those previously reported to remove T lymphocyte activity in a specific way (27, 47), only B cells were retained specifically. We have so far been unable to remove immune T cells against "conventional" antigens by any kind of immunosorbent testing T cell activity in helper systems (13, 30, 42, footnote 3, the present paper), delayed hypersensitivity studies (footnote 8), and DNA synthesis induced by antigen in vitro systems where T cells could be shown to constitute the dividing cells (footnote 6). The only system so far where specific reproducible adsorption and elution of T cells can be shown to take place (47) would seem to be on monolayers of cells differing with regard to strong histocompatiblity antigens (47, 48).

When analyzing the antigen-binding specificity of receptors on immune B cells from animals injected with hapten-autologous protein conjugates, we could separate such cells into two major groups: those with receptors for the hapten and those with specificity for the NADs on the hapten-protein conjugate. It was previously known that such NADs are quite specific for the hapten-protein conjugate studied (29, 30), and the immune response against such NADs does not follow the genetic rules of high or low response against the hapten used (29). In agreement with this, we could show that DNP-OA Sepharose beads would only remove the hapten-specific B cells from a cell population immune against DNP_6-MSA, whereas DNP_6MSA beads would remove both haptens and NAD-specific B cells (Tables V and VIII). If incubation of cells with the latter kind of beads was carried out in the presence of up to 10^{-4} M of free DNP-lys, hapten-specific B cells would not bind to the beads, but there was no decrease in the selective binding of NAD-specific cells (Table VI). It would thus seem clear that the haptenic group itself does not play any detectable direct role in the NAD epitopes or in the recognition by NAD-specific receptors. No direct numerical attempts were made on the number of anti-NAD

[8] Andersson, B. Unpublished experiments.

vs. antihapten B cells when a DNP-MSA conjugate of rather low hapten to protein ratio (5–10:1) was used, but hemagglutination titers were of comparable strength (29). When the number of haptens per protein was increased to make a $DNP_{32}MSA$ conjugate, no anti-NAD antibodies are found (29). Thus, excess of hapten in the hapten-protein conjugate would tend to interfere with the expression of NADs either by steric blocking or by actual destruction via the coupling procedure (23, 28, 29).

Due to our failures to demonstrate rosette-forming T cells in the present system and to remove specific T helper cells by incubation with antigen-coated beads, we had to resort to specificity studies of T cells in relation to T cell function. In the present system this was made possible by removing all B cells from an immune cell population via filtration through anti-immunoglobulin-coated columns (31, 32). Subsequently, such column-passed cells, deprived of specific B cells as tested by morphological and functional criteria (Tables III and IV), were used as helper cells in in vivo transfer systems. Using such tests we could demonstrate that DNP-MSA-specific T cells were induced in mice immunized with DNP_6 up to $DNP_{32}MSA$ (reference 30, footnote 5). However, the relative efficiency in helping capacity went down with increasing hapten to protein coupling ratios (above 8:1), in agreement with the studies on the B cell anti-NAD reactivity as reported above.

The specificity of DNP-MSA T cells was then studied. If the stimulating secondary antigen was a DNP-protein conjugate with no cross-reacting NADs as measured by humoral antibodies, there was also no cross-reactivity at the helper T cell level (Tables IX and X, references 29 and 30, footnote 3). However, when rat serum albumin was used as the protein for DNP coupling, a substantial cross-reactivity at the NAD level was observed (Tables XI and XII and reference 29) as well as cross-stimulation at the T helper cell level (Tables XI and XII). Whether this means that the DNP-MSA and DNP-RtSA have the same NADs activating both B and T cells in analogy with the interpretations on cross-reacting serum albumins at B and T cell levels (17, 25), or whether they are reactive against different immunogenic sites, one preferentially activating T cells and another B cells (18–22), cannot be decided at present. However, we would favor the latter in view of the fact that in agreement with findings of others (23, 24) it was not possible in the present study to demonstrate any DNP-specific helper T cells helping DNP-specific B cells in the DNP-MSA immune cells (the carrier dependence in all the present experiments and Tables VII, IX, and X), nor was it possible to demonstrate any anti-DNP antibody production trying to use DNP-skin-painted T cells as helper cells (Table IX). Even if T and B cells directed against the same immunogenic site might exist, they would seem to compete in a negative rather than collaborative way (23, 30), thus making it less likely to find B and T cells immune against the same epitope in the same immune cells population.

Summarizing, immunization in the mouse using an autologous hapten-

protein conjugate (DNP-MSA) results in the induction of immune B and T cells with specificity for the conjugate. The immune response of the B cells can be subdivided in two major groups: those reacting against the DNP group and those reactive against NADs in the mouse serum albumin introduced by the hapten coupling but with the creation of specificity governed by both the hapten and the protein (footnote 2). No binding energy for the hapten itself could be found in the antigen-binding receptors on B cells specific for such NADs. Cross-reactivity at the NAD level could be demonstrated when DNP-rat serum albumin was used as immunogen but not for other DNP-serum albumins or proteins. In the same system, no functional helper T cells specific for DNP could be detected, suggesting that also T cells had the specificity for NADs introduced by the hapten coupling without proving, however, that the NADs for B cells and the NADs for T cells have the same steric configurations. The present data would stress the role of NADs in the immune regulation of both B and T cell responses against autologous macromolecules slightly altered as to chemistry. The implications of the present findings for studies of autoimmunity and "tumor-specific" immune systems would seem obvious.

SUMMARY

Mice immunized with hapten-autologous serum albumin conjugates (DNP-mouse serum albumin) were shown to contain immune B and T cells with specificity for the conjugate. Fractionation on antigen-coated Sepharose beads showed that B cells could be subdivided in two major groups: those reacting against the haptenic group (DNP) and those reactive against the new antigenic determinants (NADs) introduced into the protein carrier by the hapten coupling. It was shown previously that humoral antibodies formed against hapten-mouse serum albumin conjugates also were directed against these two groups of antigenic determinants and that the immune response to the NADs does not follow the genetic rules of high or low response against the hapten used. All together these findings support the distinct nature of the NADs over the haptenic groups, as recognized both at the humoral and cellular level.

Absorption of mouse cells immune to hapten-autologous serum albumin conjugates on antigen-coated Sepharose beads using a variety of incubation conditions resulted in no specific retention of T cells. Therefore we had to resort to specificity studies of T cells in relation to T cell function. Relatively pure immune T cell suspensions were obtained using fractionation on anti-immunoglobulin-coated columns. DNP-MSA-specific T cells were shown to be very specific for the DNP-MSA conjugate with only one exception: they cross-reacted with antigenic determinants on DNP-rat serum albumin. As DNP-specific help was excluded in the present transfer system (as shown by the inability of cells from DNP-skin-painted mice and DNP-heterologous protein conjugate specific T cells [anti-immunoglobulin column purified] to help a

DNP-MSA response), these results demonstrate the NAD specificity of the DNP-MSA-reactive T cells. The cross-reactivity pattern of DNP-MSA-specific T cells was similar to that found for humoral anti-NAD antibodies produced against the same immunogen.

Whether B and T cells are activated by the same antigenic determinants is discussed.

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REFERENCES

1. Claman, H. N., E. A. Chaperon, and R. F. Triplett. 1966. Thymus-marrow cell combination. Synergism in antibody production. *Proc. Soc. Exp. Biol. Med.* **112:** 1167.
2. Mitchell, G. F., and J. F. A. P. Miller. 1968. Cell-to-cell interaction in the immune response. II. The source of hemolysin-foming cells in irradiated mice given bone marrow and thymus or thoracic duct lymphocytes. *J. Exp. Med.* **128:**821.
3. Taylor, R. B. 1969. Cellular co-operation in the antibody response of mice to two serum albumins: specific function of thymus cells. *Transplant. Rev.* **1:**114.
4. Mitchison, N. A., K. Rajewsky, and R. B. Taylor. 1970. Cooperation of antigenic determinants and of cells in the induction of antibodies. *In* Developmental Aspects of Antibody Formation and Structure. J. Šterzl and H. Riha, editors. Academia, Prague. 547.
5. Wigzell, H., and B. Andersson. 1969. Cell separation on antigen-coated columns. Elimination of high rate antibody-forming cells and immunological memory cells. *J. Exp. Med.* **129:**23.
6. Truffa-Bachi, P., and L. Wofsy. 1970. Specific separation of cells on affinity columns. *Proc. Natl. Acad. Sci. U.S.A.* **66:**685.
7. Wigzell, H., and O. Mäkelä. 1970. Separation of normal and immune lymphoid cells by antigen-coated columns. Antigen-binding characteristics of membrane antibodies as analyzed by hapten-protein antigens. *J. Exp. Med.* **132:**110.
8. Walters, C. S., and H. Wigzell. 1970. Demonstration of heavy and light chain antigenic determinants on the cell-bound receptor for antigen. Similarities between membrane-attached and humoral antibodies produced by the same cell. *J. Exp. Med.* **132:**1233.
9. Greaves, M. F. 1970. Biological effects of anti-immunoglobulins. Evidence of immunoglobulin receptors on T and B lymphocytes. *Transplant. Rev.* **5:**45.
10. Sell, S. 1970. Development of restrictions in the expression of immunoglobulin specificities by lymphoid cells. *Transplant. Rev.* **5:**19.
11. Miller, J. F. A. P., A. Basten, J. Sprent, and C. Cheers. 1971. Interaction between lymphocytes in immune responses. *Cell. Immunol.* **2:**469.
12. Andersson, B. 1972. Studies on antibody affinity at the cellular level. Correlation between binding properties of secreted antibody and cellular receptor for antigen on immunological memory cells. *J. Exp. Med.* **135:**312.

13. Wigzell, H. 1970. Specific fractionation of immunocompetent cells. *Transplant. Rev.* **5**:76.
14. Crone, M., K. Koch, and M. Simonsen. 1972. The elusive T cell receptor. *Transplant. Rev.* **10**:36.
15. Basten, A., J. F. A. P. Miller, N. L. Warner, and J. Pye. 1971. Specific inactivation of thymus-derived (T) and non-thymus-derived (B) lymphocytes by ^{125}I-labelled antigen. *Nat. New Biol.* **231**:104.
16. Hogg, N. M., and M. F. Greaves. 1972. Antigen-binding thymus-derived lymphocytes. II. Nature of immunoglobulin determinants. *Immunology.* **22**:967.
17. Rajewsky, K., and H. Pohlit. 1971. Specificity of helper function. *Prog. Immunol.* **1**:357.
18. Senyk, G., E. B. Williams, D. E. Nitecki, and J. W. Goodman. 1971. The functional dissection of an antigen molecule. Specificity of humoral and cellular immune responses to glucagon. *J. Exp. Med.* **133**:1294.
19. Schlossman, S. F. 1972. Antigen recognition. The specificity of T cells involved in the cellular immune response. *Transplant. Rev.* **10**:97.
20. Parish, C. R. 1972. Preferential induction of cell-mediated immunity by chemically modified sheep erythrocytes. *Eur. J. Immunol.* **2**:143.
21. Thompson, K., M. Harris, E. Benjamini, G. F. Mitchell, and M. Noble. 1972. Cellular and humoral immunity: a distinction in antigenic recognition. *Nat. New Biol.* **238**:20.
22. Schirrmacher, V., and H. Wigzell. 1972. Immune responses against native and chemically modified albumins in mice. I. Analysis of non-thymus-processed (B) and thymus-processed (T) cell responses against methylated bovine serum albumin. *J. Exp. Med.* **136**:1616.
23. Taylor, R. B., and G. M. Iverson. 1971. Hapten competition and the nature of cell co-operation in the antibody response. *Proc. R. Soc. Lond. B Biol. Sci.* **176**:393.
24. Iverson, G. M. 1971. Is an antigen bridge required for T and B cell cooperation in the immune response. *In* Cell Interactions, Third Lepetit Symposium. L. G. Silvestri, editor. North Holland Publishing Co., Amsterdam.
25. Rajewsky, K., C. Brenig, and I. Melchers. 1971. Specificity and suppression in the helper cell system. *In* Cell Interactions, Third Lepetit Symposium. L. G. Silvestri, editor. North Holland Publishing Co., Amsterdam.
26. Möller, E., and O. Mäkelä. 1971. Inhibition of anti-θ and anti-MBLA resistant rosettes by free hapten. *In* Cell Interactions, Third Lepetit Symposium. L. G. Silvestri, editor. North Holland Publishing Co., Amsterdam.
27. Davie, J. M., and W. E. Paul. 1970. Receptors on immunocompetent cells. I. Receptor specificity of cells participating in a cellular immune response. *Cell. Immunol.* **1**:404.
28. Rubin, B. 1972. Studies on the induction of antibody synthesis against sulfanilic acid in rabbits. I. Effect of the number of hapten molecules introduced in homologous protein on antibody synthesis against the hapten and the new antigenic determinants. *Eur. J. Immunol.* **2**:5.
29. Rubin, B. 1972. The immune response against hapten-autologous protein conjugates in the mouse. I. Specificity of antibodies produced during the primary

response against dinitrophenylated mouse serum albumin. *Eur. J. Immunol.* In press.

30. Rubin, B., V. Schirrmacher, and H. Wigzell. 1972. Induction of anti-hapten antibody responses against haptens conjugated to autologous and heterologous proteins. *In* Microenvironmental Aspects of Immunity. Plenum Publishing Corp., New York. 133.
31. Wigzell, H., K. G. Sundquist, and T. O. Yoshida. 1972. Separation of cells according to surface antigens by the use of antibody-coated columns. Fractionation of cells carrying immunoglobulins and blood group antigens. *Scand. J. Immunol.* **1:**75.
32. Golstein, P., H. Wigzell, H. Blomgren, and E. A. J. Svedmyr. 1972. Cells mediating specific in vitro cytotoxicity. II. Probable autonomy of thymus-processed lymphocytes (T cells) for the killing of allogeneic cells. *J. Exp. Med.* **135:**890.
33. Rubin, B. 1972. Cellular and humoral antibody production against sheep erythrocytes in AKR mice. I. The effect of antigen dose and treatment with 6-mercaptopurin on the primary immune response and on the development of immunological memory. *Acta Pathol. Microbiol. Scand. Sect. B: Microbiol. Immunol.* **79:**297.
34. Raff, M. C. 1971. Surface antigenic markers for distinguishing T and B lymphocytes in mice. *Transplant. Rev.* **6:**52.
35. Mitchison, N. A. 1971. The carrier effect in the secondary response to hapten-protein conjugates. I. Measurement of the effect with transferred cells and objections to the local environment hypothesis. *Eur. J. Immunol.* **1:**10.
36. McConnell, I., A. Munro, B. W. Gurner, and R. R. A. Coombs. 1969. Studies on actively allergized cells. I. The cyto-dynamics and morphology of rosette-forming lymph node cells in mice and inhibition of rosette-formation with antibody to mouse immunoglobulins. *Int. Arch. Allergy Appl. Immunol.* **35:**209.
37. Greaves, M. F., and E. Möller. 1970. Studies on antigen-binding cells. I. The origin of reactive cells. *Cell. Immunol.* **1:**372.
38. Takahashi, T., J. J. Mond, E. A. Carswell, and G. J. Thorbecke. 1971. Surface antigens of immunocompetent cells. II. The importance of θ and Ig bearing cells in the immune response to various antigens. *J. Immunol.* **107:**1520.
39. Mond, J. J., T. Takahashi, and G. J. Thorbecke. 1972. Surface antigens of immunocompetent cells. III. In vitro studies of the role of B and T cells in immunological memory. *J. Exp. Med.* **136:**663.
40. Siskind, G. W., and B. Benacerraf. 1969. Cell selection by antigen in the immune response. *Adv. Immunol.* **10:**1.
41. Andersson, B. 1970. Studies on the regulation of avidity at the level of the single antibody-forming cell. The effect of antigen dose and time after immunization. *J. Exp. Med.* **132:**77.
42. Wigzell, H., C. Huber, and V. Schirrmacher. 1972. Affinity fractionation of lymphoid cells according to type and function. *Acta Haematol.* (*Basel*). In press.
43. Asherson, G. L., and W. Ptak. 1968. Contact and delayed hypersisitivity in the mouse. I. Active sensitization and passive transfer. *Immunology.* **15:**405.
44. Ada, G. L. 1970. Antigen binding cells in tolerance and immunity. *Transplant. Rev.* **5:**105.

45. Humphrey, J. H., and H. U. Keller. 1970. Some evidence for specific interaction between immunologically competent cells and antigens. *In* Developmental Aspects of Antibody Formation and Structure. J. Šterzl and M. Riha, editors. Academia, Prague.

46. Dwyer, J. M., N. L. Warner, and I. R. Mackay. 1972. Specificity and nature of the antigen-combining sites on fetal and mature thymus lymphocytes. *J. Immunol.* **108:**1439.

47. Golstein, P., E. A. J. Svedmyr, and H. Wigzell. 1971. Cells mediating specific in vitro cytotoxicity. I. Detection of receptor-bearing lymphocytes. *J. Exp. Med.* **134:**1385.

48. Brondz, B. D., and A. E. Snegiröva. 1971. Interaction of immune lymphocytes with the mixtures of target cells possessing selected specificities of the H-2 immunizing allele. *Immunology.* **20:**457.