

SEPARATION OF NORMAL AND IMMUNE LYMPHOID CELLS BY ANTIGEN-COATED COLUMNS

ANTIGEN-BINDING CHARACTERISTICS OF MEMBRANE ANTIBODIES AS ANALYZED BY HAPTEN-PROTEIN ANTIGENS*

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Combination between immunogen and antibody molecules present on the relevant antigen-reactive cell(s) is commonly considered an early, essential step in the induction of detectable, high-rate antibody formation. Lymphocytes have been shown to display immunoglobulin-like molecules on their outer cell surface (1-3) and various methods have demonstrated a selective adsorption of antigen to the surface of a minority of the lymphoidal cell population (4-8). The increase of the number of reactive cells in such systems after immunization (4-10) and the specific inhibition of the reactions by blocking antigen (7, 8) indicate that lymphoidal cells can display antibody activity on their surfaces. Direct demonstration that these surface receptors could be linked to potential immune capacity of the cells carrying the surface antibodies has come from experiments where filtration of immunologically competent cells through antigen-coated columns was carried out (11, 12). In such experiments, specific elimination of the antigen-reactive cells directed against the antigen used for coating the column was obtained as indicated by the reactivity of the passed cells.

Since we believe that preformed cell-bound antigen-specific receptor molecules are of prime importance during the induction of immune processes, we have tried to obtain further information by using antigen-coated columns. We have applied the technique to nonimmunized lymphocytes. Emphasis was put on analysis of the size of the antigen-combining site of the receptors by the use of columns coated with different hapten-carrier complexes. Furthermore, investigations were made to explore whether cells potentially capable of producing antibodies of different immunoglobulin classes can be separated in a similar manner. Evidence for the existence of preformed receptors on potential antibody-forming cells directed against complex antigens, as well as against chemically well defined substances of haptenic nature will be presented. The theoretical implications of the findings will be discussed.

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Materials and Methods

Animals.—Inbred mice of the strains A.SW, A.BY, A/Sn, C57BL/6, DBA/2 and/or their respective F₁-hybrids were used. The mice were obtained from the breeding colony of the Department of Tumor Biology. Animals were of 2-4 months of age at the start of the experiments. Within each experiment, all animals were of the same strain and sex and of similar age.

Immunization.—The following immunogens were used: human serum albumin, HSA¹ (Kabi AB, Stockholm), bovine serum albumin, fraction V, BSA (Armour and Co., Ltd., Eastbourne, England), and ovalbumin, OA (KEBO AB, Stockholm). Hapten-protein conjugates were made using the NIP (4-hydroxy-3-iodo-5-nitrophenylacetic acid) coupled to HSA, BSA, or OA using different number of hapten groups per protein molecules as indicated in the text. In a few experiments, NIP was coupled to chicken globulin. Labeling procedures for the NIP to be attached to protein molecules via the azide form have been published (13). All primary immunizations were carried out using the immunogen emulsified in complete Freund's adjuvant (Difco, Laboratories, Inc., Detroit, Mich.) administered bilaterally into the footpads of the hind legs. Concentration of immunogen in the final emulsion varied from 0.5 mg/ml to 5 mg/ml. No significant differences were obtained using the different concentrations of antigen and the actual concentration in the isolated experiments will not be reported in the article. Secondary immune responses obtained when studying the immunological memory in transfer systems were initiated by the intravenous administration of 25 μ g of antigen, together with the relevant cells. This procedure will induce high rate antibody formation in primed cells only (14).

Serological Tests.—The concentration of humoral antibodies in the experimental animals directed against the various protein antigens was determined by the modified Farr ammonium sulfate precipitation method (15). Tagging of the various albumin antigens with an isotope was carried out using ¹³¹I (The Radiochemical Centre, Amersham, England) and the chloramine-T method (16). It should be noted that in many experiments the antibody concentrations in the sera were so low as to require the titrations to be carried out at very low concentrations of antigen during titration (0.1 μ g/ml or less) which would selectively favor the detection of antibodies with high avidity (17). Humoral antibodies against NIP were titrated using NIP-bacteriophages as targets (18) or, in one experimental system, using N¹²⁵I *P*-epsilon-amino-caproic acid (13). Determinations of 19S and 7S anti-NIP antibodies were carried out as previously reported (19). Antibody synthesis at the cellular level was explored, using the indirect hemolytic plaque assay for the detection of 7S antibody-producing cells using protein- or NIP-coated sheep erythrocytes as target cells (20). In this case, sheep erythrocytes were coated by using NIP-azide at 1 mg/ml for 40 min at room temperature. Otherwise, labeling was like for NNP (21).

Tests for "Primary Immune Response."—Test cells for "normal" primary immune response involved the lymph node (inguinal and axillary), spleen, and/or bone marrow cells which were studied, isolated or mixed, as indicated in the text. The cells were harvested from normal mice and a sample of the cells were passed through antigen-coated columns and, after counting for number and viability using trypan blue, 10⁷ cells were transferred into recipient mice of the same strain irradiated with 800 R. Conditions for X-irradiation have been reported (14). Immunogen was administered as indicated in the section on immunization and the mice were bled

¹ *Abbreviations used in this paper:* BSA, bovine serum albumin, fraction V; HSA, human serum albumin; MEM, minimal essential medium; NIP, 4-hydroxy-3-iodo-5-nitrophenylacetic acid; OA, ovalbumin; PFC, plaque-forming cells.

from the retroorbital plexus at different time intervals thereafter and the individual sera titrated for antibody content. Each group of experimental animals consisted of 4–8 mice.

Tests for Immunological Memory.—Test cells for immunological memory involved either popliteal lymph node cells or spleen cells or a mixture of both cell types with no essential difference in the experimental outcome. The cells were obtained from immunized animals between 1 and 14 months after primary immunization. Samples of the cell populations to be analyzed were filtered through antigen-coated columns and were counted and transferred into recipients X-irradiated with 400 R (14). Contact with bead-attached antigen will in itself not trigger memory cells into high-rate antibody synthesis (11). Antigen was administered to the recipient mice as reported in the section on immunization and the mice were bled within 10 to 20 days after transfer and boosting and the individual sera titrated for antibody content. Each experimental group consisted of 4–8 mice.

Antigen-Coated Bead Columns for Filtration of Antigen-Reactive Cells.—A detailed description of the bead column technique has been published (11). In the present article, minor modifications have been introduced, such as the use of taller columns (1.5 × 150 cm) when fractionating normal lymphoid cells for immunological reactivity, whereas 1.5 × 80 cm was used for “immune” cells. This increase in height was found necessary to evoke significant separation of normal lymphoid cells. This may be caused by the average low avidity for antigen of the cell-bound receptor on normal lymphoid cells; the columns will preferentially retain cells carrying receptors of high binding strength for the antigen.² In the present article, Degalan V 26 particles (Degussa Wolfgang AG, Hanau am Main, Germany) were used throughout for the provision of beads for antigen coating (11). The cell separation was carried out at 4°C using columns with antigen-labeled beads which had been washed as to contain no demonstrable free antigen in the columnar fluid. Cellular sieving through the columns was allowed to take place at a rate of approximately 1–2 ml/min and passed and retained cells were collected as previously reported (11). Using tall columns (150 cm), approximately 50% of the cells applied to the column were retained for “mechanical” reasons. Unless otherwise stated, retained cells were eluted by careful mechanical shaking of the beads in a glass vessel, but in a series of experiments reported in this article, specific elution was attempted by adding free hapten or complete immunogen to the column beads *after* specific retention of the antigen-reactive cells had taken place.

All cell suspensions were prepared using Eagle's minimal essential medium (MEM) Grand Island Biological Co., Grand Island, N.Y. at 4°C and MEM was used throughout the column separations.

RESULTS

Elimination of Potential Antibody-Forming Cells against Protein Antigens by Passage through Antigen-Coated Columns.—If normal lymphoid cells with potential capacity to produce humoral antibodies would display this characteristic through antigen-specific receptors on the cell surface they should, under suitable conditions, be retained on the relevant antigen-coated column, as previously demonstrated with immune cells (11). As the avidity of the receptor on the immune cell can be shown to influence the relative likelihood of specific retention of passing cells,¹ columns 150 cm high were used in the

² Andersson, B., and H. Wigzell. Analysis of the avidity of cell-bound antibody using cellular fractionation by antigen-coated columns. Manuscript to be published.

present experiments to exert a more prolonged specific "selective pressure" distance on cells filtrated through the column. 10^7 of the passing cells, the mechanically eluted cells or retained cells and the control cells were subsequently transferred to lethally X-irradiated animals, followed by immunization via

TABLE I
Separation of Immunologically Reactive Normal Lymphoidal Cells by Passage through Antigen-Coated Columns. Reactivity against Protein Antigens

Exp.	Cells*	Column†	Immuno- nogen(s)‡	Day	Anti-OA¶	Anti-BSA¶	Anti-OA¶ Anti-BSA	Relative reacti- vity**
1	C	—	NIP-BSA +OA	13	-0.478 ± 0.083	-1.075 ± 0.039	0.596	100
	P	OA	"	"	All negative	-1.029 ± 0.136	<0.038	<27
	P	NIP-BSA	"	"	-0.832 ± 0.037	All negative	>1.168	>373
	BM	—	"	"	All negative	All negative		
2	C	—	NIP-BSA +OA	20	0.692 ± 0.106	-0.702 ± 0.110	1.393 ± 0.120	100
	P	NIP-BSA	"	"	0.688 ± 0.164	-1.080 ± 0.078	1.750 ± 0.192	228
	R	"	"	"	0.370 ± 0.205	-0.915 ± 0.209	1.225 ± 0.107	68
3	C	—	NIP-BSA +OA	22	1.113 ± 0.169	0.214 ± 0.304	0.898	100
	P	BSA	"	"	1.252 ± 0.181	All negative	>2.251	>2260
4	C	—	NIP-BSA +OA	21	0.763 ± 0.081	-0.437 ± 0.141	1.199 ± 0.114	100
	P	OA	"	"	0.455 ± 0.175	-0.116 ± 0.166	0.576 ± 0.244	24
	P	NIP-BSA	"	"	0.226 ± 0.111	-1.777 ± 0.094	2.302 ± 0.159	1270
	BM	—	"	"	-0.400 ± 0.304	All negative		
5	C	—	NIP-BSA +OA	14	-0.221 ± 0.070	-0.866 ± 0.171	0.664 ± 0.154	100
	P	NIP-BSA	"	"	-0.371 ± 0.045	-1.951 ± 0.020	1.580 ± 0.095	823
	C	—	"	19	0.687 ± 0.131	0.029 ± 0.117	0.664 ± 0.169	100
	P	OA	"	"	-0.239 ± 0.081	-0.441 ± 0.112	0.202 ± 0.149	35
	P	NIP-BSA	"	"	0.224 ± 0.189	-1.296 ± 0.123	1.393 ± 0.235	536
	C	—	"	28	1.309 ± 0.071	1.173 ± 0.124	0.136 ± 0.120	100
	P	OA	"	"	0.703 ± 0.221	0.847 ± 0.174	-0.145 ± 0.253	52
	P	NIP-BSA	"	"	1.264 ± 0.192	0.577 ± 0.121	0.686 ± 0.243	355

* C, control cells. P, passed cells. R, retained cells. BM, bone marrow cells. For composition of the cell population, see text.

† Degalan, 1.5 x 150 cm. Coated with OA or NIP₁₀-BSA.

‡ Immunization was carried out in Freund's complete adjuvant using NIP₁₀-BSA and OA at 2 mg/ml.

|| Day of bleeding after cell transfer and immunization.

¶ Antibody titers expressed in log₁₀ numbers of mean \pm standard error of the mean (for antibody titrations see Materials and Methods).

** Relative reactivity = anti-OA:anti-BSA, as compared to the ratio of the control group (100%).

footpads with protein antigen(s) in complete Freund's adjuvant (see Materials and Methods). The cells comprised in most experiments of a mixture of normal lymph node, spleen and bone marrow cells, with a relative ratio of 15–25% lymph node cells, 55–65% spleen cells, and 15–25% bone marrow cells. In

some experiments control groups were included receiving bone marrow cells only. Bleedings were carried out at different days after cellular transfer and the antigen-binding capacities of the individual sera against the different antigens were determined using the Farr assay (15). The results obtained are shown in Table I and demonstrate significant specific elimination in the passed cells of cells potentially reactive against the antigen used for coating the column, whereas a minor specific increase (1.43) was observed in the retained, mechanically eluted cells (Experiment 2). A total of 15 experiments were carried out, yielding 7 with highly significant specific separation ($P < 0.001$)

TABLE II
Separation of Immunologically Reactive Normal Lymphoidal Cells by Passage through Antigen-Coated Columns. Separation According to Hapten Specificity

Exp.	Cells*	Column†	Im-munogen‡	Day	Anti-OA¶	Anti-NIP¶	$\frac{\text{NIP}¶}{\text{OA}}$	Relative reactivity**
1	C	—	NIP-OA	20	-0.588 ± 0.141	5.165 ± 0.183	5.753 ± 0.097	% 100
	P	NIP-BSA	"	"	-0.573 ± 0.065	4.420 ± 0.251	4.993 ± 0.206	17
	P	BSA	"	"	-0.641 ± 0.183	5.233 ± 0.214	5.874 ± 0.169	132
2	C	—	NIP-OA	20	0.673 ± 0.107	5.544 ± 0.103	4.871 ± 0.101	100
	P	NIP-BSA	"	"	0.588 ± 0.083	4.483 ± 0.124	3.892 ± 0.174	11
	R	"	"	"	0.544 ± 0.140	5.498 ± 0.173	4.954 ± 0.162	121

* C, control cells. P, passed cells. 10^7 cells per animal after column fractionation.

† Degalan, 1.5×150 cm. Coated with NIP₁₀-BSA or BSA.

‡ Immunization was carried out in Freund's complete adjuvant using NIP₁₁-OA at 2 mg/ml.

|| Day of bleeding after cell transfer and immunization.

¶ Antibody titers expressed in log₁₀-numbers of the mean \pm standard error of the mean. (For antibody titrations, see Materials and Methods).

**Relative reactivity = anti-NIP:anti-OA as compared to the ratio of the control group (100%).

as observed in the passed cells, 4 with "suggestive" specific elimination, whereas 4 experiments yielded no detectable specific reduction.

Separation of Normal, Anti-Hapten (NIP)-Reactive Cells by Passage through NIP-Coated Columns.—The above reported findings demonstrate the presence of surface-attached antibodies on normal, potential antibody-producing cells reactive against protein antigens. Due to the complexity of the antigens used, the data are not informative as to the binding characteristics of the receptor for anything else but the specificity. Separation of immunologically reactive cells recognizing well defined haptenic groups would allow estimates as to similarities or dissimilarities between the size of the antigen-combining site of the membrane receptor as compared to that of the humoral antibody.

In this series of experiments, normal lymphoidal cells were tested for predetermined receptors to the synthetic hapten NIP by filtration through columns coated with NIP conjugated to carrier A followed by transfer into

lethally X-irradiated recipients and immunization with NIP conjugated to carrier B. Filtrations through columns coated with carrier A alone were run in parallel to ensure that carriers A and B are not cross-reactive where a removal of anti-carrier reactive cells might simulate a removal of potential anti-hapten antibody-forming cells (22). Also in this system long columns were used (150×1.5 cm) to ensure a long travelling distance for the cells, or increased selective pressure distance like that for the separation of normal, anti-protein antibody-producing cells. The results are shown in Table II and again clearly demonstrate that normal lymphoidal cells, this time assessed for potential reactivity against an isolated haptenic group, NIP, can be selectively retained when filtered through a NIP-coated column. Passage through columns

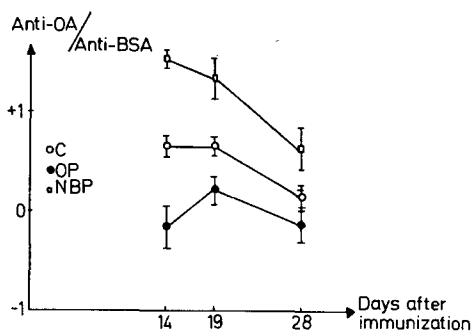


FIG. 1. 800 R irradiated A/Sn.x C57BL/6 F₁ mice received 10^7 control cells (C), 10^7 passed cells through an OA-coated column, (OP) or 10^7 cells passed through a NIP₁₀-BSA column (NBP). All animals were immunized with a mixture of NIP₁₀-BSA and OA in Freund's complete adjuvant at a final concentration of 2 mg/ml. Anti-OA/Anti-BSA ratios expressed in log₁₀ units. Mean \pm SE of means of six individual sera in each group.

coated with carrier A alone had no detectable impact on the potential anti-NIP reactivity. In this system, 15 experiments were carried out, yielding 6 with highly significant anti-NIP separation ($P < 0.001$), 4 with suggested separation, whereas in 5 experiments no detectable separation of potential anti-NIP immune reactivity was observed.

All experiments included transfer of cells into lethally irradiated recipients without including thymectomy. In view of this fact, it is not surprising that the rate of recovery from this specific immunosuppression is relatively rapid as shown in Figs. 1 and 2 and is with regard to the protein antigens virtually complete within 4 wk. Comparative rates of escape from immunological paralysis induced in vivo in nonthymectomized mice have been reported (23).

Separation of Immune, Anti-NIP Cells by Passage through NIP-Coated Columns.—In the first set of experiments immune anti-NIP cells were ana-

lyzed as to their tendency to become selectively retained when filtered through a NIP-coated column. Anti-NIP cells, obtained from animals immunized with NIP-carrier A immunogen, were allowed to filter through columns coated with NIP-carrier B or with carrier B alone. Carriers A and B were chosen as to exclude, as far as possible, serological cross-reactivity, and as a further con-

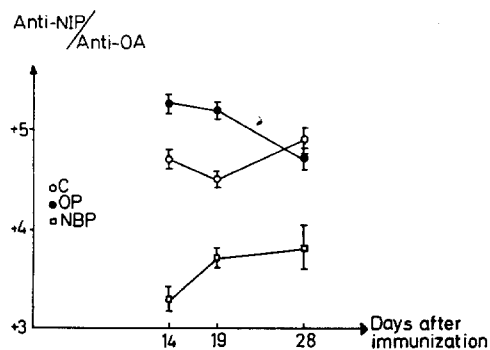


FIG. 2. The same group of experimental animals as in Fig. 1. Anti-NIP:anti-OA ratio expressed in \log_{10} units. Mean \pm SE of the means of six individual sera in each group.

TABLE III

Separation of Anti-Hapten and Anti-Protein PFC by Hapten-Protein-Coated Columns

Exp.	Cells*	Immunogen†	Column‡	NIP-PFC	HSA-PFC	OA-PFC	NIP-PFC/OA-PFC	HSA-PFC/OA-PFC	NIP-PFC/HSA-PFC
1	C	NIP-OA+HSA	—	81	209	61	1.35	3.48	0.39
	P	"	NIP-HSA	20	28	51	0.40	0.56	0.71
2	C	NIP-OA+HSA	—	79	237	69	1.13	3.39	0.33
	P	"	NIP-HSA	5	6	31	0.17	0.20	0.83
3	C	NIP-HSA+OA	—	102	191	396	0.26	0.48	0.53
	P	"	NIP-OA	6	78	7	0.86	11.14	0.08

* C, control cells. P, passed cells.

† Donors were immunized with Freund's complete adjuvant containing 2 mg/ml of NIP₁₁-OA and HSA or 2 mg/ml of NIP₁₀-HSA and OA.

‡ Degalan bead columns, 80 × 1.5 cm, coated with NIP₁₀-HSA or NIP₁₁-OA as indicated.

|| PFC expressed as PFC/10⁶ cells. Each figure represents the mean of two plates.

control for this, the control columns coated with B alone were included. In most experiments, immune cells against a third antigen, C, were admixed to the immune anti-NIP-carrier A cell population before starting column fractionation. Subsequent to column filtration, the cells were either analyzed immediately for their content of plaque-forming cells (PFC) against NIP and carrier A and C antigen or tested for the immunological memory capacity in a transfer system. The results of the two test systems (PFC or memory) are shown in Table III and IV and show that immune anti-NIP cells can be selectively

retained by NIP-coated columns when present either as PFC and/or as memory cells. Passage through columns coated with carrier B alone on the other hand had no impact on the anti-NIP reactivity.

Further evidence that the immune anti-NIP cells will have the NIP group as the immunodominant group for the surface-attached antibody receptors comes from several sets of experiments. If experiments were carried out as described above, but included the presence of free NIP-aminocaproic acid in the column fluid during cellular separation, complete blocking of the selective

TABLE IV
Separation of Immunological Memory Cells. Separation According to Hapten Specificity

Exp.	Cells*	Column†	Im- munogen‡	anti-BSA	anti-OA	anti-NIP	Anti-NIP Anti-OA	Anti-NIP Anti-BSA
1	C	—	NIP-OA	—	0.245 ± 0.017	5.808 ± 0.084	5.562 ± 0.051	—
	P	NIP-BSA	“	—	0.103 ± 0.029	4.223 ± 0.219	4.119 ± 0.203	—
	P	BSA	“	—	0.210 ± 0.036	6.034 ± 0.071	5.823 ± 0.079	—
	C	—	NIP-BSA	1.419 ± 0.077	—	5.803 ± 0.106	—	4.388 ± 0.079
	P	NIP-OA	“	0.666 ± 0.151	—	4.143 ± 0.212	—	3.486 ± 0.259
	P	OA	“	1.174 ± 0.069	—	5.060 ± 0.261	—	3.886 ± 0.280
2	C	—	NIP-CG + BSA	1.047 ± 0.164	—	6.580 ± 0.095	—	5.533 ± 0.189
	P	NIP-HSA	“	0.771 ± 0.141	—	4.799 ± 0.236	—	4.028 ± 0.263
	P	HSA	“	0.673 ± 0.194	—	6.533 ± 0.098	—	5.860 ± 0.168
3	C	—	NIP-CG + BSA	1.623 ± 0.036	—	6.235 ± 0.065	—	4.611 ± 0.106
	P	NIP-HSA	“	0.837 ± 0.208	—	3.795 ± 0.170	—	2.958 ± 0.236
	R	“	“	1.170 ± 0.219	—	5.466 ± 0.237	—	4.296 ± 0.197
	P	HSA	“	0.660 ± 0.241	—	6.139 ± 0.069	—	5.478 ± 0.201
	R	“	“	1.001 ± 0.256	—	5.873 ± 0.190	—	4.873 ± 0.267

* C, controls. P, passed cells. R, retained cells. Memory test carried as described in Materials and Methods.

† Degalan, 1.5 × 80 cm. Coated with NIP₁₀-HSA, NIP₁₀-BSA, NIP₁₁-OA, BSA, HSA, or OA.

‡ Immunogen, antigen used for primary immunization, as well as for the secondary response. For details see Materials and Methods. NIP-CG, NIP₁₅-chicken globulin.

|| Antigen-binding capacity against the respective antigens as described in Materials and Methods. Figures denote geometric mean ± standard error of the mean, all expressed in log₁₀ numbers.

anti-NIP retention of the NIP-coated column could be obtained, as shown in Table V. This affected PFC and memory cells to a similar extent. NIP-aminocaproic acid was chosen as blocker, as attachment of NIP to proteins using the azide form is considered primarily to take place via the free amino group of lysine (13). Attempts to demonstrate carrier-specific reactivity on the immune anti-NIP cell itself have also failed as exemplified in Table VI where anti-NIP-BSA immune cells with admixed anti-OA cells were allowed to sieve through a BSA-coated column. In all experiments, a highly significant retention of BSA-PFC was recorded, whereas NIP-PFC and OA-PFC filtered through to the very same extent (NIP-PFC/OA-PFC ratios in passed cells ranging from 0.73 to 1.39, as compared to control cells = 1.00). Thus, no indi-

cation of carrier reactivity within the NIP-reactive receptor attached to the immune anti-NIP cells could be demonstrated in accordance with the binding characteristics of the humoral anti-NIP antibodies (24, 25).

Separation of Normal and Immune Anti-NIP Cells of 19S or 7S Type by NIP-Protein-Coated Columns.—Previous studies on the separation of immune antibody-forming cells on antigen-coated columns have primarily analyzed the impact within the 7S system (11). It is easy to assess the relative proportions of 19S and 7S antibodies directed against NIP within the same serum sample by combined use of free hapten inhibition vs. 2-mercaptoethanol sensitivity (19). The experimental design for separation of normal or immune

TABLE V
*Blocking of Elimination of Immune Anti-NIP Cells when Filtered through NIP-Coated Columns
Effect of Presence of Free NIP-Aminocaproic Acid in the Columnar Fluid*

Cells*	Column†	Free NIP-aminocaproic acid‡	NIP-PFC	OA-PFC	NIP/OA PFC	Anti-NIP¶	Anti-OA¶	$\frac{\text{anti-NIP}¶}{\text{anti-OA}}$
C	—	—	426	247	1.72	-0.310 ± 0.146	-0.356 ± 0.089	0.046 ± 0.129
P	NIP ₂₁ -BSA	—	67	167	0.40	All negative	-0.526 ± 0.248	<1.526
C	—	30 $\mu\text{g/ml}$	396	307	1.29	-0.476 ± 0.328	-0.431 ± 0.074	-0.045 ± 0.382
P	NIP ₂₁ -BSA	“	208	102	2.04	-1.102 ± 0.254	-0.894 ± 0.175	-0.208 ± 0.359

* C, control cells. P, passed cells. Cells a mixture of regional lymph node and spleen cells immunized with NIP₂₁-OA.

† Column, Degalan V26, 80 cm, coated with NIP₂₁-BSA.

‡ Concentration of free NIP-aminocaproic acid during passage through column. Control cells incubated with the same concentration for the same time. Cells three times washed before tested.

|| PFC, arithmetic mean of PFC/10⁶ cells as derived from two plates for each figure.

¶ Antigen-binding capacity of sera against OA or NIP as assessed in Materials and Methods. Figures denote mean \pm standard error of the mean, all expressed in log₁₀ units. Negative animals in anti-NIP = less than 0.000-2 in ABC.

anti-NIP cells was as previously described and some of the experimental groups previously reported were included in this 19S-7S survey. The results are shown in Table VII and demonstrate that both 19S and 7S anti-NIP production were selectively reduced by filtration of cells through a NIP-coated column. This was found when using both normal and immune anti-NIP cell populations. The trend of the NIP-passed groups to display an increased 19S:7S ratio, as compared to the control groups of the same day does not mean a selective elimination of 7S vs. 19S cells by the columns, as the 19S:7S ratio will change with time and total anti-NIP titers in a highly predictable manner. Thus, if plotting 19S:7S ratios vs. total-anti-NIP titers within the same experiment, both passed and control groups will be on the same line, where the NIP-passed cells being lower in total-anti-NIP titer still display the expected 19S:7S ratio for that titer. As seen from the Table VII, the passed cells in

TABLE VI

Attempts to Demonstrate Carrier Specificity within the NIP-Reactive Site on NIP-PFC as Assessed by Filtration through Carrier-Coated Columns

Cells*	Immunogen†	Column‡	NIP-PFC	OA-PFC	BSA-PFC	BSA-PFC/ OA-PFC‡	NIP-PFC/ OA-PFC‡
C	NIP-BSA + OA	—	103	115	378	3.29 (1.00)	0.90 (1.00)
P	“	BSA	57	87	37	0.43 (0.13)	0.66 (0.73)
C	“	—	211	97	518	5.34 (1.00)	2.18 (1.00)
P	“	BSA	69	32	59	1.84 (0.34)	2.16 (0.99)
C	“	—	132	146	195	1.34 (1.00)	0.91 (1.00)
P	“	BSA	95	75	6	0.08 (0.06)	1.27 (1.39)

* C, control cells. P, passed cells. Three separate experiments were performed.

† NIP-BSA = NIP₅-BSA. A mixture of NIP₅-BSA + OA, 2 mg/ml of each in Freund's complete adjuvant (see Materials and Methods).

‡ Degalan V26 bead columns, 1.5 × 80 cm, coated with BSA.

|| PFC expressed as PFC/10⁶ cells plated. Each figure represents mean of two plates.

¶ First figures = absolute figures. Figures within brackets = relative values as compared to C = 1.00.

TABLE VII

Separation of Normal and Immune Anti-NIP Cells on NIP-Coated Columns. The Effect on Potential Capacity to Produce 19S and 7S Anti-NIP Antibodies

Exp.	Cells*	Immunogen†	Column‡	19S Anti-NIP	7S Anti-NIP	19S Anti-NIP/7S Anti-NIP
1	C, N, day 20	NIP-BSA + OA	—	4.670 ± 0.104	4.541 ± 0.100	0.129 ± 0.111
	P, N, “	“	NIP-BSA	3.729 ± 0.048	3.370 ± 0.138	0.359 ± 0.109
	C, N, “	NIP-OA	—	5.201 ± 0.114	5.167 ± 0.140	0.034 ± 0.144
	P, N, “	“	NIP-BSA	4.035 ± 0.150	4.195 ± 0.133	-0.160 ± 0.159
2	C, I, day 20	NIP-CG + OA	—	5.346 ± 0.187	6.601 ± 0.096	-1.255 ± 0.124
	P, I, “	“	HSA	5.293 ± 0.167	6.574 ± 0.158	-1.281 ± 0.106
	P, I, “	“	NIP-BSA	3.935 ± 0.149	4.671 ± 0.276	-0.736 ± 0.245
3	C, d, day 14	NIP-BSA + OA	—	4.240 ± 0.103	3.995 ± 0.051	0.245 ± 0.082
	P, N, “	“	NIP-BSA	2.790 ± 0.088	2.334 ± 0.117	0.456 ± 0.079
	C, N, day 19	“	—	4.776 ± 0.091	4.860 ± 0.103	-0.084 ± 0.098
	P, N, “	“	NIP-BSA	3.728 ± 0.085	3.459 ± 0.182	0.268 ± 0.110
	C, N, day 28	“	—	5.413 ± 0.158	6.202 ± 0.090	-0.789 ± 0.144
	P, N, “	“	NIP-BSA	4.387 ± 0.144	4.731 ± 0.187	-0.344 ± 0.311

* C, control cells. P, passed cells. N, normal. I, immune cells. Day stands for day of bleeding after transfer.

† Immunogen = NIP-BSA = NIP₁₀-BSA. NIP-CG = NIP₁₁-chicken globulin. Immunization of normal cells or tests for immunological memory was carried out as indicated in Materials and Methods.

‡ Degalan bead columns, 80 × 1.5 cm when using immune cells and 150 × 1.5 cm when using normal cells.

|| Anti-NIP as assessed by NIP-phage inactivation. Geometric means ± standard error of the mean in log₁₀-units.

experiment 3 have a 19S:7S ratio, suggesting a maturation period of antibody formation lagging approximately 7 days after the control cells.

Attempts to Elute Already Attached Anti-NIP Cells Retained on NIP-Coated Columns by the Use of Free NIP-Aminocaproic Acid.—If anti-NIP reactive cells retained by a NIP-protein coated column remain attached owing to the binding between bead-attached antigen and cellular receptors for the antigen, the presence of free antigen in the environment might act as a specific eluant for the anti-NIP cells. Immune anti-NIP cells were allowed to filter through NIP-coated columns in the way previously described. After the peak of the passed cells had been collected, an additional 100 ml of MEM were allowed to sieve through the column, reducing the number of cells still being eluted at the bottom to a very low figure ($<10^4$ /ml). After this, approximately 100 ml of MEM containing 10^{-3} M NIP-aminocaproic acid were allowed to fill the column and to stand at 4°C for 30 min (in one experiment the latter procedure was allowed to take place at room temperature). Subsequent to this, the flow rate was put at approximately 2 ml per min and further MEM containing NIP-aminocaproic acid was applied on top of the column. The first 200 ml of fluid coming out at the bottom of the columns were collected, the cells were washed thrice and assayed for immunological memory against NIP, as well as against another antigen not used on the column. Incubation of immune anti-NIP cells for this period of time with 10^{-3} M NIP-aminocaproic acid has been found to leave no detectable impact on the anti-NIP antibody response of these cells when tested in a memory system giving the secondary dose of antigen in vivo (26). The total yield of eluted cells using this procedure was about $0.5-3 \times 10^6$ cells and similar number of cells were eluted from normal serum-coated columns through which anti-NIP immune cells had been filtered. A total of four experiments were carried out and in no experiment did the eluted cells carry an increased relative reactivity against NIP, as compared to the memory against the other antigens. Thus, it was not possible to specifically elute already attached immune cells by the use of free antigen, whereas presence of free antigen before attachment could be shown to function as a specific blocking agent for the removing capacity of antigen-coated columns (11). The suggested explanation for the former finding will be presented in the Discussion.

DISCUSSION

In this article we have presented evidence that normal, antigen-reactive lymphoidal cells display their potential immunological reactivity by preformed antibody receptors on the outer cell surface. This was shown by the selective retention of such cells when being filtered through antigen-coated bead columns in the way previously demonstrated for immune cells (11)

and for normal bone marrow cells (12, 27, 28). The column fractionation could be shown to produce a cellular population being very similar in immunological reactivity to one being induced to become immunologically tolerant by "classical" means (23). Recovery from the specific unresponsiveness of the filtered cells could be shown to take place within a matter of a few weeks *in vivo*, being close to completeness within 4-6 wk after transfer into lethally X-irradiated recipients. It should be recalled that these transfers were made into otherwise intact animals and that the fate of such cells when transferred into thymectomized, irradiated recipients yet has to be established.

Furthermore, in the present system working with either normal or immune cells, we could show that the cell-bound antibody has the capacity to recognize isolated antigenic sites on an immunogenic molecule as analyzed by the use of hapten-protein complexes. Here, normal cells or cells obtained by immunization with the hapten NIP attached to carrier protein A would show selective elimination of the anti-NIP cells if filtered through a NIP-protein B-coated column (also, protein B-reactive cells will be retained) whereas, conversely, only anti-protein A cells would be retained if the cells were sieved through an A-coated column. Evidence that the anti-NIP reactive cell-bound receptor recognize the NIP as an immunodominant group comes from experiments where the retention of high-rate antibody-forming or memory cells directed against NIP by NIP-coated columns could be specifically blocked by including free NIP-epsilon-aminocaproic acid in the columnar fluid during cellular fractionation (NIP is considered to bind primarily in the azide form via the free aminogroup on lysine (13).

Antibodies directed against haptens have been shown to contain additional, specific binding forces for the carrier molecule, this being specifically pronounced when using certain haptens such as DNP (29). Immunization with hapten coupled to a carrier with a high positive charge will preferentially lead to the production of anti-hapten antibodies with a relatively negative charge (30, 31) suggesting that the cell-bound antibody having to combine with immunogen before switching on high-rate antibody formation might display similar binding characteristics. In the NIP system representing another hapten system, antibodies to NIP show a better binding for NIP-aminocaproic acid than for NIP alone (24), suggesting that the antigen-binding site at least includes the first amino acid to which the hapten is most commonly attached (13), but little or no true "carrier"-specificity has been found to be displayed by anti-NIP antibodies (25). Yet, in the same system typical carrier specificity can be demonstrated in the secondary anti-NIP immune response but these latter findings can to their major extent be explained on the basis of cellular cooperation between carrier and hapten-reactive cells (32), rather than being caused by the presence of carrier reactivity within the hapten-reactive, cellular antibody. Our analysis on this matter using carrier-coated columns

failed to demonstrate anti-carrier reactivity on the surface of high-rate antibody-forming anti-NIP cells as these cells would pass through the columns to the same extent as cells immune against other, irrelevant antigens. Thus, the cell-bound antibody, on at least this type of cell, seemingly has an antigen-binding area very similar if not identical with that of the releasable product of the cell, the humoral antibody.

The potential capacity to produce anti-NIP antibodies of 19S or 7S type were affected to a similar significant extent when filtering normal or immune anti-NIP-containing cell populations through NIP-coated columns. These data demonstrate that the two systems rely upon the induction of humoral antibody release and synthesis by the use of cell(s?) with similar binding energies per receptor molecule for the bead-attached NIP groups. Whether the critical cells that are retained by the antigen column are of thymic origin and/or belonging to the precursor lines for humoral antibody formation (33, 34) is unknown. Analogies between the binding requirements of humoral 19S and 7S anti-NIP antibodies, as compared to the necessary conditions for induction of antibody formation strongly suggest, however, that the potential IgM-producing system has preformed IgM-receptors differing in antigen-binding qualities from those IgG receptors supposedly present on the potential IgG high-rate producing cells (25). Further evidence that the potential antibody-synthesizing cell has preformed surface receptors with a chemistry similar to that of the eventual humoral product of the cell comes from blocking experiments using immunoglobulin class-specific antisera in column separation experiments. In such experiments, presence of anti- γ 1 antiserum could be shown to selectively let through potential γ 1 anti-NIP producing cells through a NIP-coated columns, while retaining the γ 2a cells, whereas the reverse was found when using anti- γ 2a antiserum as blocking agent.³

The present data on the retention of anti-NIP cells by NIP-coated columns all suggest that the specific forces are provided for by cell surface antibody molecules, having binding characteristics for NIP similar to those of humoral anti-NIP antibodies. It thus seemed reasonable to attempt specific elution of these retained anti-NIP cells by adding free NIP-aminocaproic acid to the columnar medium. These experiments failed to enrich anti-NIP-reactive cells and we consider them as providing further information on the mode of action of antigen-coated columns when retaining antigen-reactive cells. It has previously been found that mere changes in the flow rate of filtered cells through a normal serum-coated column will significantly alter the percentage of cells retained for nonserological reasons and, with a sufficiently reduced rate, very

³ Walters, C. S., and H. Wigzell. Demonstration of heavy chains within the cell-bound receptor for antigen. Identity between cell-bound and humoral antibodies produced by the same cell. Manuscript to be published.

few cells will pass through (11, 12). Preliminary experiments on the actual profile of antigen-reactive cells coming out at the bottom of the columns strongly suggest that if, eg. anti-A and anti-B cells are run together through an A-coated column, the anti-A cells that will "sneak through" the column will be retarded, as compared to the average anti-B cell in the same population. This would suggest that the actual retention of the antigen-reactive cells by the antigen-coated columns occurs in a two-step fashion, the first one involving the combination between surface antibodies with bead-attached antigen slowing down the rate of filtration of the relevant antigen-sensitive cells by serologically specific reactions. As a consequence of this reduction in rate of passage of the antigen-reactive cells, there will be a greater likelihood of retention, and attachment of the cells to the bead surface, whereas now the major part of the forces involved are without serological specificity. This would mean that cells once retained are, for the most part, kept at the bead surface by non-immunological forces. It would thus seem clear that using the present bead material, antigen-coated columns have their main virtues in allowing detailed analysis of cell-bound antibodies by analysis of reduction in reactivity against certain antigenic specificities in the passed cell population, whereas they are not ideal for the purification of antigen-reactive cells.

SUMMARY

Specific fractionation of immunologically competent cells derived from normal or immune animals was achieved by filtration through antigen-coated bead columns. This selective retention of the relevant reactive cells could be shown to produce a cell population, which after passage through the column would behave like a suspension rendered immunologically tolerant by "classical" means. The immunologically specific elimination of potential antibody-forming capacity of the filtered cells could be shown to affect the IgM and the IgG system to a similar extent. Analysis of the binding characteristics of the membrane antibodies responsible for this selective retention indicate striking similarities to those of the humoral antibodies produced against the antigen. Thus, the surface receptor could distinguish isolated haptenic groups on a "foreign" carrier background and the receptor of the hapten-reactive cells in the present system (high-rate antibody-forming cells against NIP) failed to combine with carrier specific determinants in analogy with the binding behavior of the serum anti-hapten antibodies. The binding of anti-hapten reactive cells to bead-attached haptens could be specifically inhibited by the presence of free hapten in the columnar fluid during cellular filtration. The results strongly suggest that the potential humoral antibody-forming cell has preformed receptors on its outer surface with binding characteristics, indicating similarity, if not identity, to those of the eventual product of the cell, the humoral antibody.

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Note Added in Proof: Recent developments using activated Bio-Gel P-6 beads as material in antigen-coated columns⁴ suggest that in these columns nonspecific attachment of cells can be drastically reduced. Preliminary experiments also suggest that here detachment of specifically adsorbed cells can be achieved by the addition of free antigen.

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