LYMPHOCYTE PROLIFERATION IN VITRO INDUCED BY HAPTEN AUTOLOGOUS PROTEIN CONJUGATES

I. A Study on the Class of Lymphocytes Responding In Vitro and on the Nature and Specificity of Their Receptors*

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Lymphocyte proliferation provides an in vitro model of cellular immunity. Thus, in guinea pigs the time-course and the specificity characteristics of this in vitro reaction mimics the delayed type hypersensitivity (DTH)[1] reaction in vivo (1–3). Both responses are characterized by a high degree of specificity, e.g., in hapten-carrier systems DTH reactions are elicited most readily by the immunizing hapten-protein conjugate (4–6). If elicitation of a DTH reaction is attempted with the hapten conjugated to a protein different from that used for immunization, a reaction can be obtained only occasionally and then with a relatively high concentration of the conjugate (7, 8). Similar observations have been reported for the in vitro lymphocyte proliferative reaction (6, 8, 9).

Immunization with hapten-autologous albumin conjugates of mice (10), guinea pigs (11, the present report), and rabbits (12) have shown that specific antibodies are formed against (*a*) the haptens, and (*b*) the new antigenic determinants (NAD) introduced by the hapten coupling reaction. In the mouse it was further demonstrated that helper T lymphocytes existed specific for the haptenic groups and for the NAD:s (13, 14). Also in the present system it will be demonstrated that the major cell population proliferating in vitro against dinitrophenylated guinea pig albumin (DNP-GPA) showed NAD specificity.

It is well known that thymus-processed lymphocytes respond to contact with antigen in vitro via an increase in DNA synthesis (3, 8, 15–17). It is not known in what

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; ChGG, chicken gamma globulin; DNP dinitrophenyl group; DTH, delayed type hypersensitivity; EAC'-RFC, cells forming rosettes with SRBC coated with 19S rabbit anti-SRBC and complement; FCA, Freund's complete adjuvant; GPA, guinea pig albumin; HSA, human serum albumin; MSA, mouse serum albumin; NAD, new antigenic determinants; NGS, normal guinea pig serum; OA, ovalbumin; PHA, phytohemagglutinin; RFC, rosette-forming cell; RSA, rabbit serum albumin; RtSA, rat serum albumin; SF, stimulation factor; SRBC, sheep erythrocytes.

way B lymphocytes participate under such conditions (18, 19). However, B-cell products have been shown to modulate the lymphoproliferative response of immune cells (15, 18, 21). In the present study we have analyzed the impact of selectively removing B lymphocytes from normal or immune lymphnode cells by filtration through anti-immunoglobulin (anti-Ig)-coated columns (14, 22, 23). The results showed that the proliferating cells had little if any conventional immunoglobulin on their membranes, i.e., they are very probably T cells.

It has been suggested that the avidity of the antigen-binding receptors on cells induced by antigen to proliferate in vitro would fluctuate, on the population level, according to dose of immunogen and time after immunization (24). This assumption was based on studies of the kinetics of the in vitro response of cells from guinea pigs immunized with low or high doses of antigen in vivo. Thus, immune cells from guinea pigs immunized either with a low dose of immunogen or harvested late after immunization responded preferentially to low doses of immunogen in vitro compared to immune cells from animals immunized either with a high dose of immunogen or harvested early after immunization (8, 25, 26). It has further been reported that the responding cells could be specifically adsorbed on antigen-coated Sepharose beads (11). In the present system, after having established the T-cell nature of the responding cells we tried to get more information about the nature of the receptors of these cells by (*a*) carrying out adsorption studies on antigen-coated Sepharose beads and (*b*) studying the dose requirement for DNA synthesis in control and anti-Ig column-passed lymphocytes assumed to be of either low or high avidity.

Our results fail to demonstrate either specific adsorption on antigen-coated Sepharose beads or significant differences in the antigen requirements of lymphocytes responding in vitro irrespective of whether the immune cells originated from guinea pigs immunized with low or high doses of immunogen or obtained early or late during the immune response. The theoretical implications of these findings will be discussed.

Materials and Methods

Animals.—Inbred guinea pigs of the Heston strain were obtained from the Department of Tumor Biology. They were 2–6 mo of age at the beginning of the experiments and both sexes used interchangeably. Blood samples were drawn from the heart. Immunization was performed subcutaneously (s.c.) in the hind foot pads and two places at the skin of the abdomen. The immunogens were emulsified with an equal volume of Freund's complete adjuvant (FCA) and 0.2 ml of the emulsion was distributed as described above.

Immunogens.—Guinea pig albumin (GPA) was prepared from normal Heston guinea pig serum (NGS) by the trichloroacetic/ethanol methods as described for mouse serum albumin (MSA) (10). Rat serum albumin (RtSA) was prepared as GPA. Bovine serum albumin (BSA), human serum albumin (HSA), ovalbumin (OA), and rabbit serum albumin (RSA) was obtained from Koch-Light Laboratories (Colnbrook, Bucks, England). DNP-conjugates of these albumins were synthesized using 2,4-dinitrophenyl-sulphonic acid (Eastman Kodak Co., Rochester, N. Y.) as coupling reagent at pH 10. The number of DNP molecules per albumin molecule (indicated as subscripts on the conjugates) was determined spectrophotometrically (27). The following DNP-conjugates were used: DNP_5BSA, DNP_5GPA, DNP_{12}-GPA, $DNP_{15}HSA$, DNP_6MSA, DNP_7OA, DNP_8RSA, and DNP_4RtSA. Phytohemagglutinin (PHA) was obtained from Wellcome Research Laboratories, Beckenham, England. The

content of each vial was dissolved in 5 ml of medium and 0.1 ml of a 1:10 dilution was added per culture.

Antisera.—Rabbit polyvalent antiguinea pig gamma globulin antiserum (anti-Ig) was prepared by hyperimmunization with Ig emulsified in FCA. Ig was prepared by ammonium sulphate precipitation and subsequent fractionation on Sephadex G 200.

Tissue Culture.—Medium F 13 (Grand Island Biological Co., Grand Island, N. Y.) containing 10% heat-inactivated (56°C for 30 min) NGS with penicillin (75 U/ml) and streptomycin (50 U/ml) were used throughout the experiments as diluent and tissue culture medium. Inguinal and popliteal lymph nodes were collected in tissue medium and single-cell suspensions were made by gentle pressing the organs through a stainless steel mesh. Further homogenization was by passage up and down through a pasteur pipette. The suspension was allowed to settle for a few minutes, the sediment discarded, and then the suspension was washed three times in medium. Sterile conditions were maintained throughout the procedure.

5×10^5–10×10^6 cells (in 0.1 ml volume) were distributed into sterile plastic tubes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) containing 1.8 ml of medium. Appropriate amounts of antigen in 0.1 ml volumes were added, and the tubes incubated for 2–5 days at 37°C in a 5% CO_2 atmosphere. Approximately 18 h before conclusion of the experiments, 1 μC of tritiated thymidine (1.0 mC/mmol, the Radiochemical Centre, Amersham, England) was added to each tube. At termination, the tubes were centrifuged at 1,500 *g* for 10 min, the supernates discarded, and the cell mass washed twice with 2 ml of ice-cold trichloroacetic acid. 0.5 ml of Hyamine was added to each tube and kept over-night at room temperature in complete darkness. The contents of the tubes were then transferred to counting vials containing 10 ml of scintillation fluid (Packard Instrument Inc. Downers Grove, Ill.), cooled to 4°C in the dark and analyzed for radioactivity (see reference 28).

Results are expressed as geometric mean cpm of triplicate cultures ($\log_{10}$ cpm $\pm$ 1 standard error of the mean [SE]) and as the stimulation factor (SF), i.e., mean cpm in antigen (or PHA)-stimulated cultures over mean cpm in cultures without antigen.

Rossette Assay.—The number of detectable antigen-binding cells was determined by the rosette assay (14, 29, 30). Target cells were sheep erythrocytes (SRBC) coated with albumins by means of bis-diazotized benzidine (12) or with DNP-ChGG (dinitrophenylated chicken gamma globulin with anti-SRBC activity [14]). The rosette assay was performed as follows: 5×10^6 lymphoid cells (in 0.1 ml) were mixed at room temperature with 0.1 ml of a 2% protein-SRBC complex. The mixture was immediately centrifuged for 10 minutes at 200 *g* and phosphate buffered saline (1/15 M, pH 7.4) was added up to 2.0 ml. The pellet was resuspended gently using a pasteur pipette. In inhibition studies lymphoid cells were incubated for 30 min at room temperature with different dilutions of inhibitor (in 0.1 ml) before addition of erythrocytes.

The number of rosette-forming cells (RFC) was determined using a hemacytometer at room temperature. A 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 50 RFC in immune cell suspensions and 10 RFC in normal or column passed suspensions were counted. The results were expressed as number of RFC per 10^6 viable lymphocytes, i.e., anti-$\log_{10}$ to the geometric mean of six–eight determinations per suspension. The concentration of inhibitor giving 50% inhibition of the number of RFC in the control suspension was taken as a measure of avidity (30).

Rosette formation performed as described above was inhibited completely in presence of rabbit anti-Ig antiserum. This finding, the change in avidity of RFC during immunization, and the relatively low concentration of DNP-lys or DNP_3OA giving 50% reduction of the DNP-RFC counts (10^{-6}-10^{-9} M and 10^{-8}-10^{-11} M, respectively) are some major arguments for considering the RFC to be made by B lymphocytes (30–32).

Determination of the number of EAC′-RFC and membrane Ig-positive lymphocytes by immunofluorescense was performed as described previously (22, 23).

Antigen-Coated Bead Columns.—DNP_5GPA was coupled to Sepharose 6B supersize (800-15.00 μ) beads (kindly donated by Pharmacia, Uppsala, Sweden) using CNBr as coupling reagent (see 14). 10 ml of medium was added to about 10 g of DNP-GPA conjugated beads, placed in a glass column (1.5 × 30 cm, K15/30, Pharmacia). 20 ml of the cell suspension to be adsorbed (5×10^7 cells/ml) was then added, beads and cells were mixed by gentle agitation, the column placed in a horizontal position at 20°C or 37°C and left for 1 or 4 h before elution of the cells. Cells were finally washed twice before cultured. Cell yield by this procedure was between 30 and 60% of the input. Sterile conditions were obtained by incubating the glass column overnight with absolute ethanol and washing extensively first with sterile saline and then with medium. DNP-GPA-conjugated Sepharose beads in sterile saline was incubated for 2–4 h at 70°C before placed in the sterile column.

Anti-Immunoglobulin-Coated Columns.—Anti-Ig columns were prepared as described previously (14, 21, 23). Glass beads 225 μ in diameter (Superbrite, Minnesota Mining and Manufacturing Co., St. Paul, Minn.) were first coated with guinea pig Ig, poured into a glass column, washed, incubated with excess of polyvalent rabbit anti-Ig anti-serum, and washed again. Such columns contain anti-Ig molecules, many of which bind to the bead attached Ig through only one of their antigen-combining sites, leaving the other free to react with cell membrane-bound Ig. Cell fractionation was carried out at room temperature using a flow rate of 2–3 ml/min. After passage the cells were washed twice before cultured. Cell recovery was 20–30% of the input. Sterile conditions were obtained as described above and by using sterile filtered reagents. Acid-treated glass beads were boiled in distilled water for 10 min and thereafter washed in sterile saline. Characterization of retained and passed cells have been discussed extensively elsewhere (14, 22, 23, 33), always demonstrating a selective and close to complete retention of B lymphocytes.

Serology.—Hemagglutination was carried out using the microtiter equipment. Titrations were performed in 1% NGS in PBS. Anti-DNP antibodies was determined using DNP_7OA-coupled SRBC (coupling reagent, glutaraldehyde [10, 12]). Antiprotein antibodies were detected by means of protein-coated SRBC. Anti-NAD antibodies were determined against $DNP_{12}GPA$-coated SRBC, the titrations performed in presence of 10^{-3}M DNP-lys in analogy with anti-NAD antibody determination in the mouse (10) or in the rabbit (12). Results are expressed as geometric means of $\log_2$ titers. Adsorption of anti-DNP-GPA antisera on DNP_7OA Sepharose bead columns, and determination of 19S/7S antibody ratios by the 2-mercaptoethanol method has been described (10, 12).

RESULTS

Characterization of the Experimental System.—As immunogens in this study we have chosen DNP-GPA and HSA. Preliminary experiments revealed these immunogens to lack cross-reactivity in the guinea pig and to be of similar immunogenic strength when tested for induction of antibody synthesis in vivo or DNA synthesis in vitro. In analogy with the DNP-MSA mouse system (14) immunization of guinea pigs with DNP-GPA induced antibody formation of two distinct specificities: (*a*) against the DNP-group and (*b*) against the NAD:s. The specificity of anti-NAD antibodies produced against DNP-GPA was tested in a hemagglutination inhibition assay using anti-DNP-GPA antisera that had been passed through DNP_7OA Sepharose columns. Fig. 1 shows that only DNP-GPA was able to inhibit the anti-NAD-GPA specific hemagglutination. Also, DNP-conjugates of albumins from related species such as mouse, rat, or rabbit did not cross-react with DNP-GPA at the NAD level.

In different experiments presented in this paper as well as unpublished results, the DNP conjugates listed in Fig. 1 have been used as heterologous conjugates in DNA synthetic experiments. Furthermore, none of these conjugates induced any significant proliferative response of DNP-GPA immune cells in vitro. (Table IV and V, Fig. 2, and unpublished experiments).

The kinetics of the proliferative response with DNP-GPA and HSA immune cells was then investigated. Keeping the culture volume constant at 2.0 ml throughout the experiments, the antigen-specific and PHA-induced prolifera-

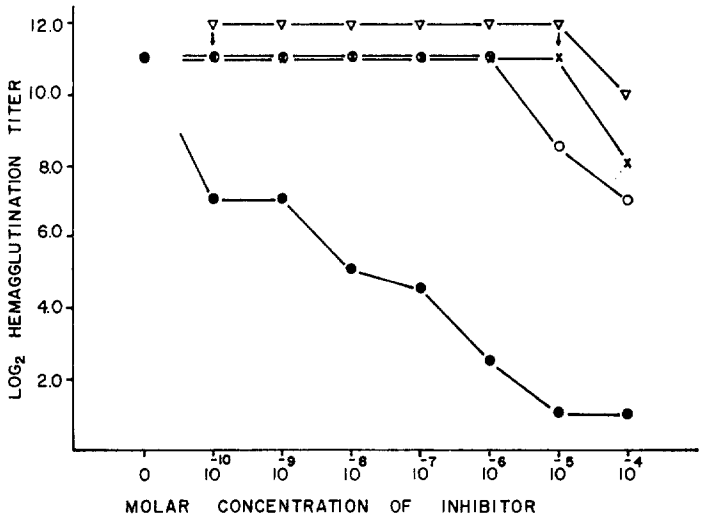

FIG. 1. Specificity of anti-NAD antibodies produced against DNP-GPA in guinea pigs. Log_2 hemagglutination titers of anti-DNP-GPA antiserum against $DNP_{12}GPA$-SRBC in the presence of 10^{-3} M DNP-Lys. Molar concentration of inhibitor was calculated on the basis of the molecular weights of the albumins (BSA, GPA, MSA, RSA, and RtSA = 69,000; OA = 40,000). DNP_4RtSA, ▽—▽; DNP_5BSA, ▽—▽; DNP_7OA, ▽—▽; DNP_5GPA, ●—●; DNP_6MSA, ○—○; DNP_8RSA, ×—×.

tive responses were tested using cell numbers per culture ranging from 5×10^5–10×10^6 immune cells. 5×10^6 immune cells per culture proved to be an optimal cell dose in the present system whereas the minimum number of cells giving a significant response was about 10^6 cells per culture (Table I). The cultures were harvested after 3 days (the last 18 h in presence of [^{3}H]thymidine), since this culture period gave the greatest difference between background and antigen induced DNA synthetic responses (Fig. 2).

The concentration of antigen in the cultures was varied from 0.02 μg to 20,000 μg per culture. Increasing responses with increasing antigen concentrations were observed with immune cells, the threshold dose of activation frequently being 0.02 μg per culture (see Fig. 2). However, antigenic stimulation of immune cells with 2,000 μg of antigen per culture gave unspecific in-

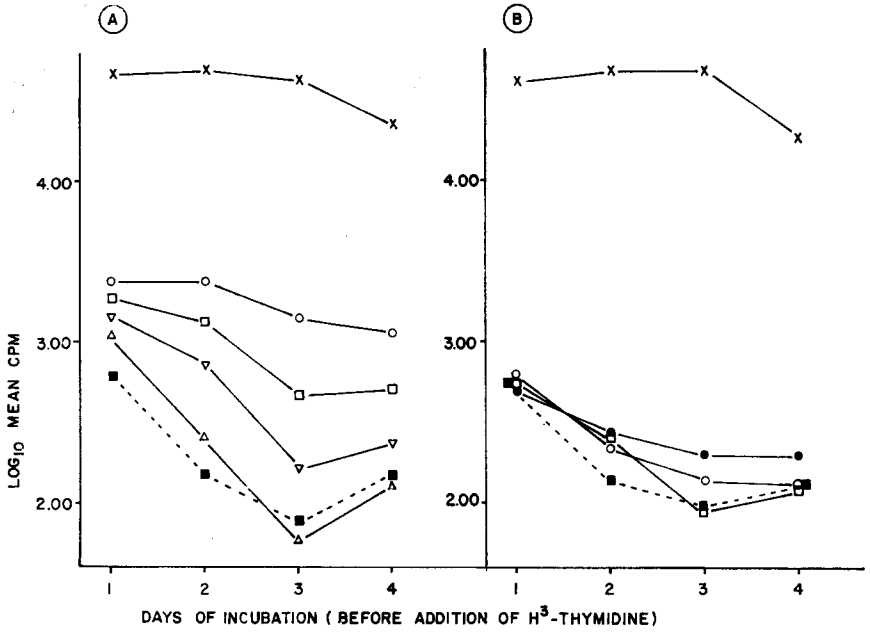

FIG. 2. Effect of the in vitro incubation time on the antigen-induced DNA synthetic response of immune (*A*) or normal (*B*) guinea pig lymph node cells. (*A*) Immune cells from animals immunized with 100 μg DNP_4GPA 49 days before culture. PHA, ×—×; 20 μg DNP_4GPA in vitro, ○—○; 2 μg DNP_4GPA, □—□; 0.2 μg DNP_4GPA, ▽—▽; 0.02 μg DNP_4GPA, △—△; without antigen, ■---■. (*B*) "Normal" cells from animals immunized with FCA 49 days before culture. PHA, ×—×; 200 μg DNP_4GPA, ●—●; 20 μg DNP_4GPA, ○—○; 2 μg DNP_4GPA, □—□; without antigen, ■---■. Stimulation with 200 μg DNP_4RtSA was included as control in this experiment. The response was very similar to that in cultures without antigen.

hibition of co-cultivated cells of another specificity. Normal cells did only make a low response to antigen at very high doses (around 20 mg/ml) using cell numbers in the range described for immune cells and incubation times as described above.[2] However, their PHA response was comparable to that of immune cells.

Immune cells from guinea pigs immunized with high (100-1,000 μg) doses of antigen and harvested relatively early after immunization may carry-over antigen from in vivo to the cultures. To test this possibility, cells from animals immunized with a low or a high dose of DNP-GPA in vivo were irradiated with 4,000 R and thereafter mixed with an equal number of nonirradiated immune cells. Stimulation of cells from low antigen dose animals by irradiated

[2] Rubin, B. and H. Wigzell. Lymphoproliferative response of normal and immune lymph node or bone marrow cells. Impact of fractionation on anti-immunoglobulin-coated columns. *In* Proc. of the 8th Leucocyte Culture Conference. Academic Press, Inc., New York. In Press.

cells from animals immunized with a high dose of antigen was looked for. No evidence for antigen carrying-over was found, however (Table II).

On the Nature of the Responding Cells.—Fractionation of normal and immune guinea pig lymphocytes on anti-Ig columns was carried out to study the nature of the responding cells in our system. Such columns have been shown to remove selectively B lymphocytes from mouse and human lymphocyte populations (14, 22, 23). In the present study we have analyzed the ability of anti-Ig columns to retain selectively (*a*) antigen-specific RFC, (*b*) EAC′-RFC and

TABLE I

*Antigen-Induced DNA Synthetic Response of DNP_4GPA Immune Cells. Effect of Cell Dilution on Relative [^{3}H]Thymidine Incorporation**

Cells‡	DNP^4GPA in vitro	Number of cells per culture§			
		1×10^6	2×10^6	5×10^6	10×10^6
I	0	1.00[a]	1.00[b]	1.00[c]	1.00[d]
	0.02 μg	0.87	1.01	2.46	2.50
	20 μg	1.55	2.51	6.30	7.08
	0.5% PHA	19.2	41.3	76.8	103
II	0	1.00[e]	1.00[f]	1.00[g]	1.00[h]
	0.02 μg	0.91	1.86	3.39	3.16
	20 μg	2.75	5.62	10.7	12.0
	0.5% PHA	20.4	43.8	75.8	67.6

* Mean cpm of triplicate cultures with antigen over mean cpm of triplicate cultures without antigen. Mean cpm in cultures without antigen: (*a*) 641; (*b*) 778; (*c*) 815; (*d*) 776; (*e*) 778; (*f*) 834; (*g*) 617; (*h*) 778.

‡ Lymph node cells from guinea pigs immunized with 1 μg (I) or 1,000 μg (II) of DNP_4 GPA 14 days before culture.

§ Immune cells diluted in 10% NGS/F13.

TABLE II

Impact of Antigen Carrying-Over by Cells Stimulated with a High Immunogen Dose in Vivo on the DNA Synthetic Response In Vitro. [^3H]Thymidine Incorporation (Log cpm)

Untreated cells per culture*		Irradiated cells per culture‡		μg DNP-GPA per culture			
				0.0	0.02	0.2	20
10×10^6 cells (1,000 μg)		—		3.706	4.062	4.383	4.800
2×10^6 cells (1,000 μg)		—		3.188	3.163	3.337	3.548
10×10^6 cells (1 μg)		—		3.557	4.253	4.399	4.566
2×10^6 cells (1 μg)		—		3.074	3.070	3.105	3.564
5×10^6 cells (1,000 μg)	+			3.673	—	—	—
5×10^6 cells (1 μg)							
5×10^6 cells (1 μg)		5×10^6 cells (1,000 μg)		3.450	—	—	—
5×10^6 cells (1,000 μg)		5×10^6 cells (1 μg)		3.365	—	—	—
		5×10^6 cells (1 μg)	+	1.908	—	—	—
		5×10^6 cells (1,000 μg)					

* Lymph node cells from guinea pigs immunized with 1 μg or 1,000 μg of DNP_5GPA 10 days before culture.

‡ Cells were irradiated with 4,000 R immediately before culture.

membrane-Ig positive cells from guinea pig lymph node cells. As can be seen in Table III, anti-Ig columns removed more than 90% of the antigen-specific RFC and more than 95% of EAC'-RFC and membrane-Ig positive cells. However, the passed cells elicited antigen-specific and PHA induced DNA synthesic responses of similar magnitude or even higher when compared to nonpassed control cells (Tables IV and V). The threshold dose of activation

TABLE III

Depletion of DNP- and SRBC-Specific RFC, Membrane Ig Positive Cells and EAC'-RFC by Anti-Ig Columns

Cells*	Target cells	Inhibitor‡	RFC/10^6 cells§	% reduction after column passage
DNP_4GPA cells:C^a	DNP-SRBC	−	2,100 ± 275	—
DNP_4GPA cells:C^a	SRBC	−	853 ± 193	—
DNP_4GPA cells:C^a	DNP-SRBC	+	1,012 ± 307	—
DNP_4GPA cells:P^a	DNP-SRBC	−	163 ± 95	92
DNP_4GPA cells:P^a	SRBC	−	90 ± 102	89
DNP_4GPA cells:P^a	DNP-SRBC	+	175 ± 78	83
DNP_4GPA cells:C^b	DNP-SRBC	−	5,750 ± 398	—
DNP_4GPA cells:C^b	SRBC	−	695 ± 197	—
DNP_4GPA cells:C^b	DNP-SRBC	+	1,080 ± 209	—
DNP_4GPA cells:P^b	DNP-SRBC	−	209 ± 111	96
DNP_4GPA cells:P^b	SRBC	−	173 ± 97	75
DNP_4GPA cells:P^b	DNP-SRBC	+	143 ± 103	86

	Ig positive cells‖ (*I*)	*EAC'-RFC*‖ (*II*)	(*I*)	(*II*)
FCA cells:C	18%	26%	—	—
FCA cells:P	1%	1%	94	96

* Lymph node cells from the experiment in Table IV: (*a*) 1 μg, and (*b*) 100 μg of immunizing antigen.

‡ 10^{-4}M of DNP-lys per 5×10^6 immune cells.

§ RFC/10^6 lymph node cells against the target cells indicated. SRBC-RFC:s are not subtracted from DNP-SRBC-RFC:s.

‖ Tested as described previously (22).

for normal as well as immune cells was not changed by passage through anti-Ig columns. A major reason for the increased relative DNA synthetic responses was the frequent drastic reduction in background DNA synthesis of the passed cells. The exact reason for this reduction is unknown but would seem linked with the column separation as such, since normal serum columns will also have this impact (28).

However, the present results indicate that purified, immune guinea pig T lymphocytes are quite capable of proliferating in vitro upon antigen stimulation in agreement with the findings of others (16, 17). If purified B lymphocytes

TABLE IV

The Antigen-Induced DNA Synthetic Response of Guinea Pig Lymph Node Cells

Antigen in vitro	Relative ^3H incorporation*			
	1 μg DNP_4GPA immune cells‡		100 μg DNP_4GPA immune cells‡	
	Control	Anti-Ig passed§[a]	Control	Anti-Ig passed§[b]
0	1.00[c] ‖	1.00[d]	1.00[e]	1.00[f]
0.2 μg DNP_4GPA	3.80	4.39	5.87	8.55
2.0 μg DNP_4GPA	6.10	14.9	10.1	16.9
20 μg DNP_4GPA	8.30	35.7	16.1	24.9
200 μg DNP_4GPA	11.8	69.1	20.4	54.5
200 μg DNP_7OA	0.75	1.02	0.71	0.93
0.5% PHA	48.9	275	100	193

* Log_{10} mean cpm in antigen stimulated cultures − log_{10} mean cpm cultures without antigen.

‡ Lymph node cells from guinea pigs immunized 16 days before stimulation in vitro.

§ Cell recovery after column passage: (*a*) 29%; (*b*) 33%.

‖ Actual cpm: (*c*) 4,950; (*d*) 832; (*e*) 2,700; (*f*) 1,333.

TABLE V

Effect of Anti-Ig Column Passage on Normal and Immune Lymph Node Cells

Antigen in vitro	Relative ^3H incorporation*			
	DNP_4GPA immune cells‡		CFA immune cells‡	
	Nonpassed	Anti-Ig passed	Nonpassed	Anti-Ig passed
0	1.00§[a]	1.00[b]	1.00[c]	1.00[d]
0.2 μg DNP_4GPA	—	11.3	—	0.78
2.0 μg DNP_4GPA	13.6	23.9	0.78	0.94
20 μg DNP_4GPA	10.2	31.8	0.69	0.97
200 μg DNP_4GPA	13.7	57.4	1.38	1.05
200 μg DNP_8RSA	0.91	0.34	0.86	0.84
0.5% PHA	630	321	176	462

* See Table I.

‡ Lymph node cells from guinea pigs immunized with 10 μg DNP_4GPA or CFA 48 days before in vitro stimulation. Cell recovery: DNP_4GPA immune cells, 38%; CFA immune cells, 34%. DNP-RFC before passage (immune), $8,278/10^6$; after passage, $231/10^6$; reduction, 97.2%. DNP-RFC (CFA immune) before passage, $813/10^6$; after passage, $193/10^6$; reduction, 76.3%.

§ Actual cpm: (*a*) = 304; (*b*) = 641; (*c*) = 870; (*d*) = 543.

could also respond under similar conditions, it was not analyzed in the present system.

Incubation of DNP-GPA Immune Cells with DNP-GPA-Sepharose Beads. Effect on DNA Synthetis.—Since the preceeding results indicated that a major part of the cells responding to antigen in vitro belonged to the T-cell lineage attempts were made to specifically adsorb these cells on DNP-GPA Sepharose

beads in order to get more information about the nature and specificity of the antigen receptor involved in the triggering of these cells (11). Immune cells from guinea pigs immunized with DNP-GPA or HSA were mixed in vitro and the cell mixture was incubated with DNP-GPA Sepharose beads for one hour at room temperature or 37°C (see Materials and Methods). Thereafter, control and adsorbed cells were tested for their ability to form rosettes with DNP- or HSA-SRBC, and for their DNA synthetic responses against DNP-GPA and HSA. One such experiment carried out at 37°C is presented in Table VI. The control mixture of cells contained 3×10^6 of each immune cell popula-

TABLE VI

Antigen-Induced DNA Synthetic Responses of Mixed DNP-GPA and HSA Immune Guinea Pig Lymph Node Cells. Effect of Adsorption on DNP-GPA Sepharose Beads*

Antigen in vitro	[^{3}H]Thymidine incorporation			
	Control cells‡		DNP-GPA Sepharose absorbtion cells‡	
	Log_{10} cpm ± SE	SF§	Log_{10} cpm ± SE	SF§
0	3.664 ± 0.048	1.00	3.223 ± 0.036	1.00
0.2 μg DNP-GPA	4.234 ± 0.048	3.72	3.789 ± 0.050	3.68
20 μg DNP-GPA	4.412 ± 0.037	5.47	3.946 ± 0.106	5.29
20 μg DNP-GPA	4.536 ± 0.060	7.35	4.311 ± 0.050	12.2
200 μg DNP-GPA	4.749 ± 0.022	12.1	4.685 ± 0.024	29.0
0.2 μg HSA	4.611 ± 0.020	8.85	4.152 ± 0.013	6.75
2 μg HSA	4.854 ± 0.058	15.5	4.389 ± 0.063	14.7
200 μg HSA	4.693 ± 0.038	19.9	4.854 ± 0.024	42.8
0.5% PHA	5.238 ± 0.123	37.5	5.293 ± 0.013	11.8

* Immune cells from guinea pigs immunized with 10 μg DNP-GPA or 100 μg HSA, 28 days before in vitro stimulation.

‡ Cell recovery after adsorption: 44% of the input. DNP-RFC and HSA-RFC before adsorption: 8,390/10^6 cells and 8,150/10^6 cells; after adsorption: 866/10^6 cells and 9,670/10^6 cells.

§ SF, stimulation factor calculated as relative [^{3}H]thymidine incorporation.

tion in order to culture suboptimal numbers of immune cells (see first section of Results). It can be seen that the DNP-GPA Sepharose beads retained about 90% of the DNP-specific RFC whereas HSA-specific RFC were, if anything, somewhat enriched. However, there was no specific adsorption of DNP-GPA specific cells able to be induced into DNA synthesis by DNP-GPA. Three more experiments (one more carried out at 37°C and two at room temperature) gave similar results. In fact, adsorption on DNP-GPA Sepharose beads seemed to give a cell suspension displaying reactivity characteristics similar to those cells passed through an anti-Ig column (Tables IV and V).

Therefore, mixed DNP-GPA and HSA immune cells were passed through either an anti-Ig column or adsorbed onto DNP-GPA Sepharose beads (room temperature). The impact of the two columns on the DNA synthetic responses

is presented in tables VII and VIII. Almost identical results were obtained, i.e., both columns had no specific impact on either the DNP-GPA or the HSA specific DNA synthetic responses (Table VII). However, the anti-Ig column removed more than 98% of all antigen specific RFC, whereas the DNP-GPA Sepharose beads removed 96% of DNP-RFC leaving the HSA specific RFC in the non-adsorbed cell suspension (Table VIII). Thus, selective removal of B cells reactive with DNP-GPA had no detectable, specific impact of the remaining T cells to respond to DNP-GPA in vitro.

Antigen Dose and Time after Immunization. Effect on the Avidity of Antigen-Specific RFC and on the Dose Requirements for Antigen-Induced DNA Synthetis.—We next studied the effect of immunogen dose in vivo and time after

TABLE VII

Impact of Passage through Anti-Ig or DNP-GPA Sepharose Columns on the DNA Synthetic Response of DNP-GPA and HSA Immune Cells

Antigen in vitro	[^{3}H]Thymidine incorporation; mixed DNP-GPA and HSA immune cells*					
	Control cells		Anti-Ig absorbtion cells		DNP-GPA Sepharose absorbtion cells‡	
	Log_{10} cpm ± SE	SF	Log_{10} cpm ± SE	SF	Log_{10} cpm ± SE	SF
0	3.282 ± 0.011	1.00	3.076 ± 0.009	1.00	2.739 ± 0.028	1.00
0.2 μg DNP-GPA	3.398 ± 0.029	1.31	3.524 ± 0.011	2.51	3.016 ± 0.029	1.89
2.0 μg DNP-GPA	3.498 ± 0.013	1.65	3.724 ± 0.017	5.30	3.246 ± 0.013	3.21
20 μg DNP-GPA	3.835 ± 0.049	3.59	3.995 ± 0.023	8.30	3.490 ± 0.015	5.63
200 μg DNP-GPA	4.228 ± 0.021	8.85	4.285 ± 0.019	16.1	3.752 ± 0.010	10.3
0.2 μg HSA	3.883 ± 0.019	4.00	3.789 ± 0.021	5.16	3.500 ± 0.020	5.76
20 μg HSA	4.045 ± 0.013	5.81	3.948 ± 0.037	8.87	3.698 ± 0.031	9.10
2000 μg HSA	4.335 ± 0.017	11.3	4.225 ± 0.023	14.1	3.876 ± 0.059	13.7
0.5% PHA	5.164 ± 0.031	76.5	5.062 ± 0.081	97.0	4.994 ± 0.044	183

* Immune cells from guinea pigs immunized with 10 μg DNP-GPA (14 days) or 100 μg HSA (28 days).

‡ Cell recovery after DNP-GPA Sepharose absorbtion, 37% of the input; after anti-Ig column passage, 21%.

TABLE VIII

Depletion of DNP- or HSA-Specific RFC on DNP-GPA Sepharose[a] or Anti-Ig-Coated Columns[b]

I.C.*	Target cells	Inhibitor‡	RFC/10^6 – SD§	% reduction of RFC after column absorbtion
(DNP-GPA + HSA)-C	DNP-SRBC	—	21,750 ± 375	—
(DNP-GPA + HSA)-C	DNP-SRBC	10^{-4} M DNP-lys	1,350 ± 318	—
(DNP-GPA + HSA)-C	HSA – SRBC	—	12,250 ± 967	—
(DNP-GPA + HSA)-C	HSA – SRBC	10^{-5} M HSA	875 ± 143	—
(DNP-GPA + HSA)-P[a]	DNP-SRBC	—	750 ± 103	96.5
(DNP-GPA + HSA)-P[a]	HSA-SRBC	—	15,250 ± 568	−24.5
(DNP-GPA + HSA)-P[b]	DNP-SRBC	—	123 ± 88	98.5
(DNP-GPA + HSA)-P[b]	HSA-SRBC	—	90 ± 105	99.3

* Lymph node cells from DNP-GPA or HSA immune guinea pigs mixed in vitro before column absorption. Cell recovery after DNP-GPA Sepharose absorbtion. (*a*) 37% of the input; after anti-Ig column passage (*b*) 21%.

‡ Molar concentration of inhibitor per 5 × 10^6 immune cells.

§ RFC/10^6 lymphoid cells against the given target cells. Geometric means ± 1 standard deviation of eight determinations per suspension.

immunization on the avidity of antigen-specific RFC and on the subsequent in vitro induction of DNA synthesis in relation to antigen concentration. Attempts were made to evaluate the influence of (*a*) presence in the cultures of B cells and antibodies (free or cell-bound) and (*b*) the number of antigen sensitive cells per total number of cells cultured, on the kinetics of the proliferative response.

Lymph node cells from guinea pigs immunized with 100 μg DNP-GPA (or HSA) 5, 10, and 20 days before harvest were tested for their ability to form DNP-specific RFC of different avidity as well as for their antigen-induced proliferative response in vitro. The following results were obtained: (*a*) the concentration of DNP-lys needed to cause 50% inhibition of the DNP-RFC decreased with time after immunization (30, 51, see Fig. 3, whereas (*b*) no change with time of preferential stimulation of DNA synthesis at low antigen concentrations in vitro was observed for any of the three cell populations (5×10^6 cells per culture), either before or after anti-Ig column passage (Fig. 4). Several experiments (a total of 19) of similar design as well as experiments where cell populations from animals immunized with a low (0.1–1.0 μg DNP-GPA or HSA) or a high (100–1,000 μg DNP-GPA or HSA) dose of antigen gave similar results: (*a*) the avidity of antigen-specific RFC increased with decreasing dose of immunizing antigen and with time after immunization (29, 31, 33), but (*b*) no preferential stimulation of DNA synthesis at low antigen concentrations in vitro was observed either with decreasing dose of immunizing antigen or with time after immunization. Also, as before (Table IV), passage

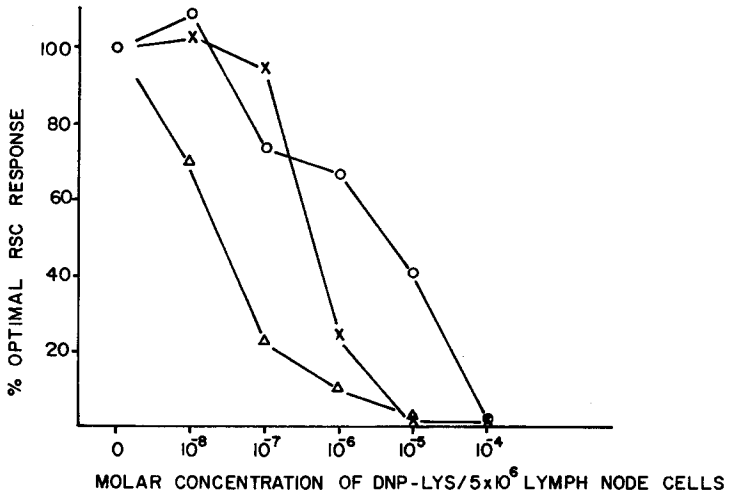

FIG. 3. Inhibition of DNP-specific RFC:s by different concentrations of DNP-lys. Rosette inhibition data of DNP_4GPA immune cells from guinea pigs immunized with 100 μg DNP_4GPA, 5, 10, or 20 days before the test. (Cells from the experiment depicted in Fig. 3). Day 5 cells, O—O; day 10 cells, X—X; day 20 cells, △—△.

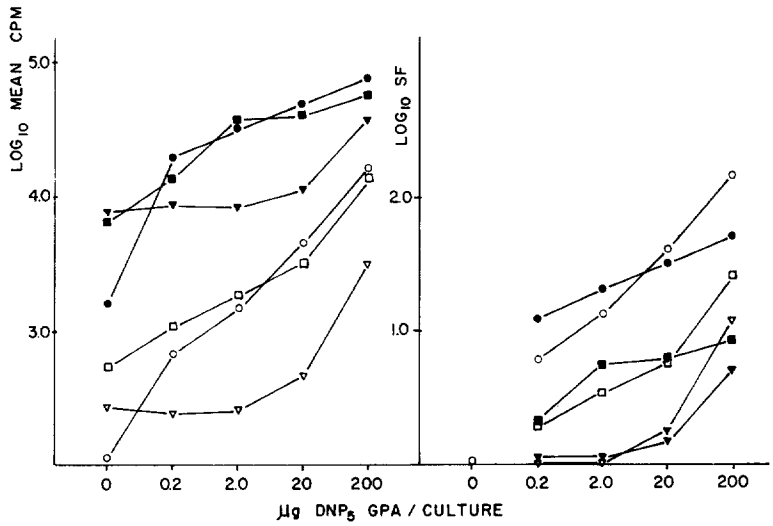

FIG. 4. Antigen-induced DNA synthetic response of DNP-GPA immune cells harvested early after immunization. Effect of anti-Ig column passage. Lymph node cells from guinea pigs immunized with 100 μg DNP_4GPA. Control cells, closed symbols; anti-Ig passed cells, open symbols. Day 5 cells (triangles), day 10 cells (circles), and day 20 cells (squares) were harvested on the same day and tested within the same experiment. Recovery after anti-Ig passage: Day 5 cells, 29%; day 10 cells, 28%; day 20 cells, 21%. Reduction of DNP-specific RFC:s day 5 cells, 90%; day 10 cells, 97.5%; day 20 cells, 98%.

through anti-Ig columns always gave relatively increased DNA synthetic responses especially at high antigen concentrations in vitro, i.e., giving DNA stimulation profiles with slopes steeper than those of nonpassed cells.

The kinetics of the DNA synthetic response of the day 5 cell population (Fig. 4) might mean (*a*) that this cell population contained relatively few antigen sensitive cells per total number of viable cells cultured and/or (*b*) that these cells have receptors of relatively low avidity. In order to distinguish between these possibilities series of experiments were carried out where the effect of immune cell dilution on the kinetics of the proliferative response of nonpassed and anti-Ig column passed cells was determined (immune cells diluted in normal [FCA immune cells] cells in order to keep the total number of cells cultured constant). The absolute DNA synthetic response of nonpassed cells decreased roughly parallel with decreasing number of immune cells cultured over the whole antigen dose range tested. As before anti-Ig column passage increased the relative responses especially at high antigen concentrations in vitro at any given number of cells cultured (Fig. 5). In only 2 out of 19 experiments mentioned above did an indication of preferential stimulation at low antigen concentrations of cells, assumed to be of high avidity, take place. However, in these two experiments the difference could be demonstrated only

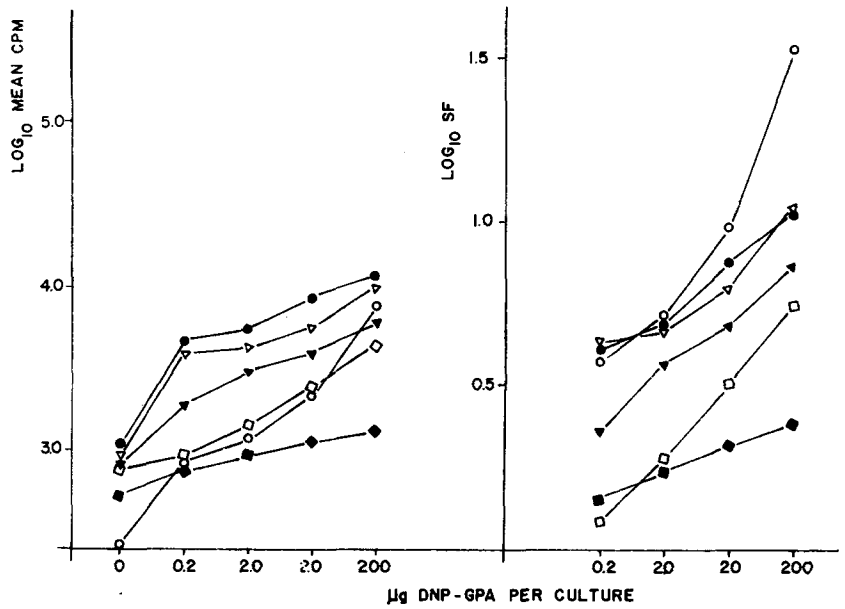

FIG. 5. Effect of cell dilution on the proliferative response of non-passed and anti-Ig column passed lymph node cells. Immune cells from guinea pigs immunized with 10 μg DNP_4GPA 28 days before in vitro stimulation. Cell recovery after anti-Ig column passage, 31%; DNP-RFC before passage, $11,780/10^6$ cells; after passage, $805/10^6$ cells; reduction, 93%. Closed symbols, nonpassed cells; open symbols, anti-Ig column passed cells. Circles, 6×10^6 cells/culture; triangles, 2×10^6 cells/culture; and squares, 0.7×10^6 cells/culture.

when high number of cells were cultured (5–10 $\times$ 10^6 cells per culture), whereas the slopes of stimulation turned identical when culturing 2×10^6 cells (Fig. 6). From the above findings a difference in the number of antigen-sensitive cells per total number of cells cultured, rather than a difference in the average avidity of the receptors on the responding cells, would seem to be a critical factor in determining the profile of antigen-reduced DNA-synthesis. Thus, we could find no evidence in the present system that a significant change in the functional avidity of immune T cells will occur during immunization (30).

DISCUSSION

It is by now well established that B and T lymphocytes are capable of expressing autonomous, immune specificity. Both groups of lymphocytes can be shown to express their immune specificity via antigen-binding surface receptors, but whereas this is easily documented on B lymphocytes (32, 34–36) as evidenced by physical separation via immunosorbants, similar attempts encounter difficulties when it comes to T cells. Using chemically prepared immunosorbants it has thus been possible to remove B but not T cells by most

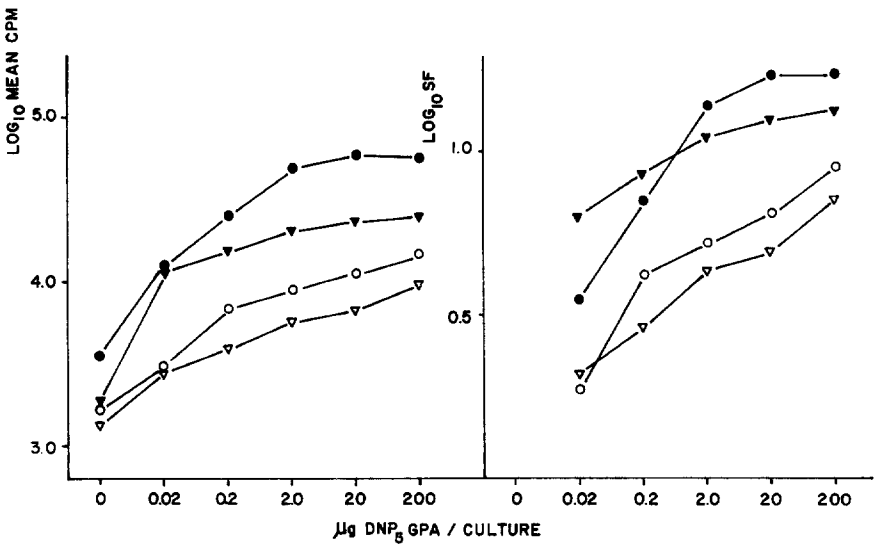

FIG. 6. Antigen-induced DNA synthetic responses of DNP-GPA immune cells in vitro. Effect of immune cell dilution. Lymph node cells from guinea pigs immunized with 1,000 μg DNP-GPA (circles) or 1 μg DNP-GPA (triangles), 10 days before in vitro stimulation. 10×10^6 cells per culture, closed symbols; 2×10^6 cells per culture, open symbols. The latter cells were adjusted to 10×10^6 cells per culture by addition of 8×10^6 normal (FCA immune) lymph node cells.

workers (see 14), with one reported exception (11). On the other hand, when using histocompatibility systems, T lymphocytes reactive against foreign transplantation antigens can be shown to bind selectively to the relevant monolayer of foreign cells (37–39). Also, suicide experiments using radioactive antigens have documented selective removal of T-cell function as a result of incubation with antigen (40, 41), thereby further documenting the actual existence of antigen-binding receptors on immunocompetent T lymphocytes. In order to study the fine specificity of the T-lymphocyte receptor for antigen it would be highly desirable to be able to use chemically defined immunosorbants instead of monolayers and we thus tried to repeat the positive results suggesting specific T-cell removal by chemically defined immunosorbants (11).

Our first approach was to make reasonably sure that the system was indeed measuring T lymphocytes reacting with antigen and not other cells such as B lymphocytes, which have been reported to proliferate in vitro in response to specific antigen (18, 19, 28, 41). This we achieved by filtration of the cells through anti-Ig columns, known from before in other species combinations to be highly efficient in removing B lymphocytes (as well as adhesive cells [33]) from the passing cells (14, 22, 23). In the present article we include results documenting the efficiency of the columns in the guinea pig system as evidenced by removal of surface Ig-positive cells as well as cells capable of binding C3-

coated erythrocytes, both characteristics of B cells (43–45). At the same time, virtually all detectable antigen-binding cells (as measured by rosette formation) were removed by filtration through anti-Ig columns. In the mouse, such removal of rosette-forming cells if achieved by either rosette sedimentation (46–50) or filtration through anti-Ig columns (13, 14, and Wigzell, unpublished observations) will only remove the B-cell activity against these erythrocytes whilst leaving the T-cell system intact. One would thus assume that specific immunocompetent B cells can react against the relevant erythrocytes by rosette formation whereas there exist no evidence that e.g., specific helper T cells against the erythrocytes can be removed by rosette techniques.

In the present article lymphoid cells from guinea pigs immune against a given hapten-protein antigen when filtered through anti-Ig columns could be shown to retain their capacity to respond against the antigen in vitro as evidenced by, in fact, increased DNA synthesis. This is in agreement with the results of others in this species (16, 17), merely demonstrating that "pure" immune T lymphocytes can react with the antigen to become switched into cell division. As in fact, cells passed through such a column demonstrated a highly enriched, relative capacity to be switched into DNA synthesis this would suggest that in the present system even in a heterologous cellular population T lymphocytes would be the dominating cells with regard to antigen-induced proliferation in vitro. Furthermore, when cells were incubated with antigen-coated beads of relevant specificity, a close to complete removal of specific B lymphocytes was observed with no corresponding, selective change in DNA synthesis in response to this antigen. Our attempts to repeat the results of others using close to identical experimental conditions (11) failed to confirm that cells capable of being switched into increased DNA synthesis (that is, most likely T lymphocytes) could be selectively removed by antigen-coated beads. Whenever we observed a reduction in DNA synthesis after incubation with the immunosorbant, we would always find that a similar reduction was taking place if now testing the cells against a second, noncross-reactive antigen. As our beads at the same time were highly efficient in removing specific B lymphocytes we can only conclude that our immunosorbant material was really functioning as an efficient immunosorbant, but only for the B and not the T lymphocytes. We have no explanation for our failure to confirm earlier positive reports in a very similar system (11).

In immune systems displaying memory on the B cell level, there is normally an increase in avidity with time after immunization, being most easily explained on the basis of laws of thermodynamics (24). Whereas memory and increase in avidity is pronounced within the IgG system (8, 9, 24, 30, 32, 50), both memory and increases in avidity are less significant (52–54) or sometimes absent (55–59) in the IgM compartment. In fact, thymus-independent humoral antibody synthesis has never been found to display any increase in avidity with time after immunization and there is normally also very poor

memory in such systems (55, 58, 59). With regard to affinity or avidity on the T cell level reports do vary (8, 25, 26, 30). If antigen-induced DNA synthesis using thymus dependent antigens in in vitro short term experiments is considered to primarily involve T cell division reports exist suggesting that a low dose of antigen will primarily induce immune T cells with a higher avidity than those evoked by a higher dose of the same immunogen (8, 25). With increasing time after immunization some workers have reported an increase in avidity of T cells (as measured by lower threshold doses of antigen-induced DNA synthesis in vitro) (25, 26) whereas others report that such an increase does not take place (8, 62). None of these earlier workers used purified T lymphocytes but a heterogenous cell population obtained from the respective lymphoid organs. In view of the fact that B cells and their products (15–18, 20, 21) as well as macrophages (20, 60–62) can be shown to be of great importance for the resultant DNA synthesis induced by antigen in vitro using heterogenous cell populations, we considered it wise to repeat the above experiments using more defined cellular systems. Here we were taking advantage of more recent fractionation procedures allowing the production of relatively "pure" T-cell populations (14, 22, 23). Using such an approach we have been unable to demonstrate any increase in the sensitivity in T-lymphocyte population toward antigen-induced DNA synthesis in relation to reduction in antigen dose or time after immunization. The profiles of DNA synthesis in relation to antigen concentration in vitro could be shown to be primarily dependent upon the number of antigen-reactive cells in the population studied. Significant changes in the shape of the profiles were obtained by merely diluting the immune cells in normal (FCA immune) cells, or by changing the number of cells per culture. In such a way, the same immune cells could be shown to under certain conditions yield a DNA synthetic profile induced by antigen that would be consistent with the presence of a large proportion of high avidity T cells, whereas using another number of cells the curve would now be that of an expected low avidity population. From this we would conclude that estimates on affinity of T-cell receptors based on antigen-induced DNA synthesis would require extremely stringently controlled experimental conditions to be really informative. Using heterogenous cells or purified T lymphocytes and testing the immune cells obtained at different time intervals after immunization for antigen-induced DNA synthesis using varying antigen doses and cell numbers we have failed to find any evidence of a significant increase in functional "avidity" of specific T lymphocytes. In view of what has been stated above, however, we would like to emphasize the requirements for caution when it comes to quantitative analysis of antigen-provoked cellular proliferation in vitro.

SUMMARY

Immune lymph node cells from guinea pigs respond to soluble antigen in vitro by an increase in DNA synthesis. Optimal conditions for this prolifera-

tive response were studied in the present article. Under such conditions, immune cells showed increasing responses with increasing antigen concentration in vitro, the threshold dose of activation frequently being as low as 0.02 μg per culture. In contrast, normal lymph node cells (from FCA-stimulated animals) did only respond to antigen at very high doses (20 mg/culture), and immune cell dilution studies could be performed in normal cells without changing the kinetics of the antigen specific response of immune cells.

Fractionation on anti-Ig columns indicated that purified, immune T lymphocytes were quite capable of proliferating in vitro upon antigen stimulation. However, our attempts to adsorb the proliferating cells onto chemically defined immunoadsorbants failed despite the fact that immune B cells (as measured by the rosette assay) were retained almost completely by such a procedure.

Purified, immune T lymphocytes from guinea pigs immunized with different antigen concentrations in vivo and/or obtained at different times after immunization were tested for a differential sensitivity toward antigen-induced DNA synthesis in vitro. However, we were not able to demonstrate any regular increase in sensitivity to antigen in vitro, and if found, it seemed to be more dependent upon the number of antigen reactive cells in the population studied rather than upon differences in the average avidity of the receptors on the cells proliferating in vitro. The results in the present article are discussed in relation to current knowledge and hypotheses on T-lymphocyte receptors.

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