SPECIFICITY OF CELLULAR IMMUNE RESPONSES

ANTIGEN CONCENTRATION DEPENDENCE OF STIMULATION OF DNA SYNTHESIS IN VITRO BY SPECIFICALLY SENSITIZED CELLS, AS AN EXPRESSION OF THE BINDING CHARACTERISTICS OF CELLULAR ANTIBODY*

BY WILLIAM E. PAUL,‡ M.D., GREGORY W. SISKIND, M.D., AND BARUJ BENACERRAF, M.D.

(From the Departments of Pathology and Medicine, New York University School of Medicine, New York 10016)

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Cellular immune responses are characterized by a high degree of specificity. Thus, in haptenic systems delayed hypersensitivity reactions are elicited most readily by the immunizing hapten-protein conjugate (1, 2). If elicitation of a delayed 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 only with a relatively high concentration of the conjugate (3). Similarly, secondary responses in haptenic systems can be obtained early in the course of immunization only with the immunizing antigen (4). Late in the course of immunization, modest secondary responses can be evoked by hapten conjugated to proteins other than that used for initial immunization (hapten–heterologous protein conjugates) (5, 6). These late secondary responses elicited with hapten–heterologous protein conjugates appear to involve the stimulation of a subpopulation of cells forming antibody of exceedingly high affinity for the hapten itself (7).

The specificity of cellular interactions with antigen implies the presence of a cell-associated receptor capable of reacting with antigen on the basis of structural complementarity. Furthermore, the selective pressure of antigen observed in the secondary response (7), in tolerance induction (8, 9), and in the increase in antibody affinity during the immune response (10), strongly suggests that this cell-associated receptor is virtually identical to the antibody capable of being secreted by that cell or its progeny, at least with regard to binding characteristics. Thus far, the quantitative study of the specificity of cellular immune responses, which is required to understand cell-antigen interactions on a thermodynamic level, has been seriously impeded by the need to utilize the intact animal to obtain these reactions.

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The recent proposals of in vitro models of cellular immune responses (11–14) make possible a more precise study of the specificity of these reactions. The antigen-stimulated incorporation of tritiated thymidine into DNA by lymphoid cells from immunized animals (11, 12) was selected for use in studying the effects of antigen dose and of time after immunization on cellular immunity. In addition, the relative capacity of the immunizing hapten-protein conjugate and of hapten conjugated to other proteins to stimulate increased thymidine incorporation into DNA was investigated. The affinity for hapten of the serum antibody present at the time of study of cellular immunity was measured in order to compare these two interrelated facets of the immune response.

Materials and Methods

Proteins and Dinitrophenyl-Protein Conjugates.—Guinea pig albumin (GPA) was prepared by starch block electrophoresis as previously described (2). Ovalbumin (OVA) (5 × recrystallized) was purchased from Pentex, Inc., Kankakee, Ill.; calf skin gelatin (GEL) was obtained from Eastman Organic Chemicals, Rochester, N.Y.; bovine fibrinogen (BF) and bovine gamma globulin (BGG) were purchased from Armour Pharmaceutical Company, Kankakee, Ill.; hemocyanin (LiH), prepared from Limulus polyphemus, was the gift of Dr. Z. Ovary. 2,4-dinitrophenylated (DNP) proteins were prepared by the reaction of 2,4-dinitrofluorobenzene (Eastman Organic Chemicals) with proteins under alkaline conditions in a manner analogous to that described by Benacerraf and Levine (2). The following conjugates were prepared: $DNP_{40}GPA$, $DNP_{53}BGG$, $DNP_{19}OVA$, and $DNP_{42}GEL$. $DNP_{24}LiH$ was the gift of Dr. Z. Ovary. Subscripts refer to the average number of hapten groups per mole for GPA, BGG, and OVA and per 100,000 molecular weight units for GEL and LiH. Tritiated DNP-GPA (^{3}H-DNP-GPA) was similarly prepared by the reaction of tritiated 2,4-dinitrofluorobenzene (156 mc/mM) (New England Nuclear Corp., Boston, Mass.) with GPA.

Other Reagents.—*N*,ε-2,4-dinitrophenyl-L-lysine (ε-DNP-L-lysine) was purchased from Cyclo Chemical Corp., Los Angeles, Calif. 2,4-dinitrophenol (DNP-OH) was obtained from Mann Research Laboratories, N. Y. and was crystallized from hot water prior to use. Eagles' minimum essential medium (MEM), penicillin, and glutamine were purchased from Grand Island Biological Company, Grand Island, N.Y.

Immunization of Guinea Pigs.—Hartley strain guinea pigs were immunized with antigen emulsified in complete Freund's adjuvant. 0.1 ml of emulsion was injected in each of the four foot-pads. The adjuvant was prepared by adding killed *Mycobacteria tuberculosis* $H_{37}RA$ (Difco Laboratories, Detroit, Mich.) to incomplete Freund's adjuvant (Difco) in a final concentration of 2 mg/ml.

Skin Tests.—Skin tests were performed by the intradermal injection of 0.1 ml of antigen-containing solution. The reactions were examined at 6 and at 24 hr after testing. Delayed reactions were evaluated on the basis of the diameter of induration 24 hr after injection.

Cultures.—Guinea pigs were anesthetized with ether and bled from the heart. The axillary, inguinal, and popliteal lymph nodes were removed under sterile conditions and rinsed three times in MEM. They were then trimmed of fat and teased into a cell suspension in MEM containing 15% pooled normal guinea pig serum and penicillin (50 units/ml). Large tissue aggregates were allowed to settle and the supernatant fluid was centrifuged at 800 rpm for 10 min. The cells were resuspended in MEM containing serum and penicillin and brought to a concentration of 2×10^7 nucleated cells per milliliter. 0.5 ml (1×10^7 cells) was placed in 12 × 75 mm plastic culture tubes (Falcon Plastics, Los Angeles, Calif.); 1.0 ml of MEM containing serum, penicillin, and the appropriate amount of antigen and/or hapten was then added to tubes. Culture tubes were usually set up in triplicate. Tubes were incubated at 37°C in an

atmosphere of 5% CO_2-95% air. After 24 hr, 0.1 ml of a solution of tritiated thymidine (^{3}H-TdR) (10 μc/20 μg/ml) was added to each tube and incubation was continued for an additional 24 hr. At the end of this time, cells were collected by centrifugation, washed with 1.0 ml of phosphate-buffered saline (PBS: 0.15 M NaCl, 0.01 M sodium phosphate buffer, pH 7.6), and frozen and thawed three times as a cell pellet. Cold 0.5 M perchloric acid (PCA) was added and, after being held at 4°C overnight, the precipitate was washed three times with cold 0.25 M PCA, once with ethanol-ether (3:1), and once with ether. The precipitate was suspended in 0.5 M PCA, and incubated at 70°C for 2 hr in order to hydrolyze the DNA. An equal volume of 0.5 M KOH was added and the mixture held overnight at 4°C to allow potassium perchlorate to precipitate. After centrifugation, an aliquot of the supernatant fluid containing the hydrolyzed DNA was added to 15 ml of Bray's solution (15) and radioactivity measured in a Tri-Carb liquid scintillation counter (Packard Instrument Co., Downers Grove, Ill.). This method of preparation of DNA hydrolysates for measurement of radioactivity is essentially that described by Reich et al. (16).

Presence of Radioactive Antigen in Culture Fluid.—Hartley guinea pigs were immunized, in a manner similar to that described above, with ^{3}H-DNP-GPA. Upon sacrifice, the lymph node cells of these animals were treated as described above for the preparation of cultures. Aliquots of culture fluid were taken for the measurement of radioactivity in order to estimate the antigen persisting from the initial immunizing injection. An internal standard of ^{3}H-DNP-GPA was utilized in order to correct for any quenching.

Purification of Antibody.—Anti-DNP antibodies were specifically purified (17) by 0.1 M DNP-OH extraction of washed specific precipitates, formed at equivalence with DNP-BF. DNP-BF was precipitated from the extract with streptomycin sulfate (35 mg/ml) and DNP-OH was removed by dialysis and by Dowex 1-X8 chromatography.

Measurement of Antibody-Binding Affinity by Fluorescence Quenching.—Affinities of purified anti-DNP antibodies for DNP ligands were calculated from the quenching of antibody fluorescence upon binding of hapten (10). Titrations were carried out by the addition of small aliquots of DNP-ligand at 2.9×10^{-6} M to 2 ml samples containing 30 μg of antibody per milliliter in PBS. The diminution of antibody fluorescence at 350 mμ was measured in a thermostated Aminco-Bowman spectrophotofluorometer at 26°C. The calculations of results were performed as previously described (18) using a Control Data computer made available by the Section on Communication Sciences, Department of Neurosurgery, New York University Medical Center.

RESULTS

Delayed Hypersensitivity Reactions.—Guinea pigs immunized with 50 μg of DNP-GPA were skin tested with 2 μg and with 10 μg of the immunizing antigen and with 10 μg and 200 μg of both DNP-OVA and DNP-GEL.

At 1 wk after immunization most guinea pigs responded to 2 μg of DNP-GPA with significant delayed skin reactions and all responded to 10 μg, usually with marked reactions (Table I). 10 μg of DNP-OVA and of DNP-GEL were ineffective in eliciting responses in these guinea pigs but 200 μg caused a significant response in 9 of 10 animals tested with DNP-OVA and in 2 of 10 tested with DNP-GEL. Another group of animals was tested 2 wk after immunization; all displayed delayed reactions to 2 and to 10 μg of DNP-GPA. 10 μg of DNP-OVA caused a significant response in one of five guinea pigs and 10 μg of DNP-GEL resulted in a significant response in four of five guinea pigs. A dose of 200 μg of either DNP-OVA or DNP-GEL elicited delayed reactions in all animals tested. In normal guinea pigs, 200 μg of these DNP proteins produced

no significant induration. It appears that at 2 wk after immunization 200 μg of DNP-OVA or DNP-GEL evoked delayed skin responses roughly equivalent to those elicited by 2 μg of the immunizing antigen, DNP-GPA. In addition, the responsiveness to DNP-OVA and to DNP-GEL seems to have increased somewhat between 1 and 2 wk after immunization with DNP-GPA. These results

TABLE I

Delayed Reactions to DNP-Proteins in DNP-GPA–Immunized Guinea Pigs

Immunizing antigen	Time after immunization	Diameter of response*	Test antigens					
			DNP-GPA		DNP-OVA		DNP-GEL	
			2 μg	10 μg	10 μg	200 μg	10 μg	200 μg
	wk	*mm*						
DNP-GPA (50 μg)	1	>20	0‡	3	0	0	0	0
		16–20	0	5	0	0	0	0
		11–15	8	2	0	2	0	2
		6–10	1	0	0	7	0	0
		0–5	1	0	10	1	10	8
DNP-GPA (50 μg)	2	>20	0	1	0	1	0	0
		16–20	0	3	0	0	0	0
		11–15	2	1	0	2	0	3
		6–10	3	0	1	2	4	2
		0–5	0	0	4	0	1	0
			200 μg		200 μg		200 μg	
Adjuvant alone		>20	0		0		0	
		16–20	0		0		0	
		11–15	0		0		0	
		6–10	0		0		0	
		0–5	5		5		5	

* Diameter of response 24 hr after intradermal injection, expressed as millimeters of induration.

‡ Number of guinea pigs displaying a delayed response within the specified range.

obtained with in vivo delayed reactions encouraged us to feel that study of cellular reactivity in an in vitro system where more precise quantitation of responses was possible might prove fruitful. Thus, the following studies were undertaken.

The Effect of DNP-Protein in Lymph Node Cell Cultures Prepared from Adjuvant Immunized Guinea Pigs.—In order to establish that the antigen stimulation of thymidine uptake which was observed in lymphoid cell cultures prepared from immunized animals was specific, the effect of various DNP-proteins on ^{3}H-TdR incorporation into DNA in cultures derived from animals immunized

with Freund's adjuvant alone was measured. Incorporation of ^{3}H-TdR into DNA is expressed as a value relative to the incorporation of ^{3}H-TdR in antigen-free cultures prepared from the same animal. The latter were assigned a value of 1.00. At concentrations of 10^{+2} μg/ml neither DNP-LiH, DNP-OVA, nor DNP-GPA caused a measurable increase in thymidine incorporation into DNA in cultures prepared from guinea pigs immunized with adjuvant alone (Table II). Furthermore, the constancy of these values indicates that these measurements can be obtained with good precision.

The Effect of Immunizing Dose of DNP-GPA.—Guinea pigs were immunized

TABLE II

In Vitro ^{3}H-TdR Incorporation in Response to DNP-Proteins in Cultures Derived From Guinea Pigs Immunized with Freund's Adjuvant

Test antigen	DNP-LiH	DNP-OVA	DNP-GPA
Concentration in culture fluid	100 μg/ml	100 μg/ml	100 μg/ml
Incorporation of ^{3}H-TdR relative to control cultures	1.29*	1.16	1.19
	1.21	1.20	1.02
	1.10	1.16	
	0.70	1.01	
	1.01	0.80	
	0.97		
	1.00		
Mean	1.04	1.07	1.10
SD	0.19	0.16	

* Results are expressed on a scale relative to ^{3}H-TdR incorporation into DNA in cultures, from the same guinea pig, incubated in the absence of antigen. ^{3}H-TdR incorporation in such cultures was assigned a value of 1.00.

with either 1, 50, or 3000 μg of DNP-GPA and cultures were established 7–19 days later. The effect of DNP-GPA in the tissue culture fluid at concentrations of from 10^{-4} to 10^{+2} μg/ml on ^{3}H-TdR incorporation into DNA in these cultures was measured. Fig. 1 presents the results of these experiments. Each curve represents an average of the results obtained in seven or more individual experiments.

These in vitro responses to antigen are characterized by great sensitivity. Thus, in several instances, a significant increment in ^{3}H-TdR incorporation into DNA is stimulated by a concentration of 10^{-4} μg/ml of DNP-GPA. Furthermore, the degree of stimulation is related to the concentration of antigen present over a wide range of antigen concentrations. In many cultures, an antigen concentration of 10^{+2} μg/ml of DNP-GPA caused a greater stimulation of ^{3}H-TdR

incorporation than did 10^0 μg/ml. This implies a great heterogeneity in responsiveness of the sensitive cell populations studied in these experiments, which is quite in keeping with the established heterogeneity of binding affinity of serum antibody. It is obvious that the immunizing dose of antigen has a profound effect on the sensitivity of lymphoid cell cultures to stimulation by anti-

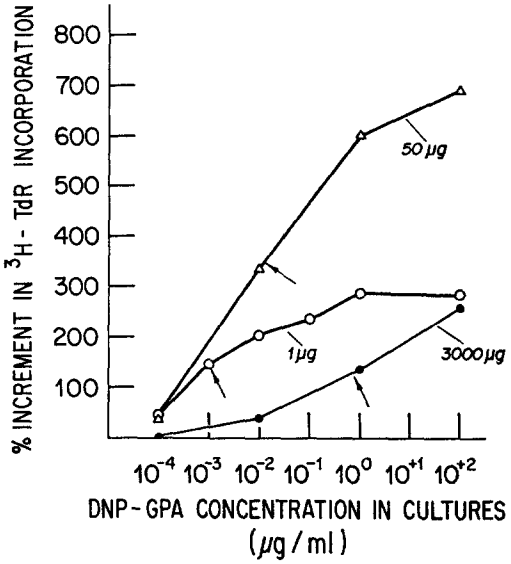

FIG. 1. Concentration-response curves for stimulation of in vitro ^{3}H-TdR incorporation into DNA as a function of immunizing dose. Guinea pigs were immunized with either 1, 50, or 3000 μg of DNP-GPA. Cultures were established with concentrations of DNP-GPA ranging from 0 to 10^{+2} μg/ml. ^{3}H-TdR incorporation is expressed as the percentage increment over that in control cultures, derived from the same animal, incubated in the absence of antigen. The arrows indicate the concentrations of DNP-GPA which stimulate 50% of the maximum observed increment in ^{3}H-TdR incorporation. Each curve represents combined data from seven or more individual experiments.

gen. Cultures derived from guinea pigs immunized with 1 μg of DNP-GPA 13–15 days earlier showed increased DNA synthesis in the presence of DNP-GPA at concentrations of 10^{-4}–10^{-3} μg/ml and displayed no additional increment in response at concentrations greater than 10^0 μg/ml. The maximum response obtainable in these cultures has a mean value of about 4 times the level of DNA synthesis in unstimulated cultures. The antigen concentration required to produce 50% of maximum observed stimulation of ^{3}H-TdR incorporation will be used to compare the relative sensitivity to antigen stimulation of lymphoid cultures obtained from guinea pigs immunized with various doses of DNP-GPA. In cultures derived from guinea pigs immunized with 1 μg of DNP-GPA

13–15 days earlier, this "50% stimulation" concentration was $\sim 10^{-3}$ μg/ml.

Lymph node cultures derived from guinea pigs immunized with 50 μg of DNP-GPA 7–19 days earlier displayed a sensitivity to low concentrations of antigen similar to that of cultures derived from animals immunized with 1 μg. However, the maximum response (eightfold over unstimulated cultures), which was considerably greater than in cultures derived from guinea pigs immunized with 1 μg, was elicited with a concentration of 10^{+2} μg/ml. 50% of maximum stimulation of DNA synthesis was obtained with 10^{-2} μg/ml, a concentration 10 times greater than that required for cultures derived from animals immunized with 1 μg of DNP-GPA. Thus cultures from guinea pigs immunized with 50 μg

TABLE III

Culture Fluid Concentration of ^{3}H-DNP-GPA as a Result of Initial Immunization

Immunizing dose	Time after immunization	^{3}H-DNP-GPA concentration in culture fluid
μg	*days*	*ng/ml**
1	7	<0.2
1	11	<0.06
50	7	0.2
50	11	<0.06
	11	<0.06
1360	10	0.36
	10	0.8
	10	0.4

*ng, nanograms (10^{-3} μg).

of DNP-GPA differed from those derived from guinea pigs immunized with 1 μg in that a greater concentration of antigen was required for maximum stimulation. This implies that a portion of the responsive cell population bound antigen relatively poorly and thus required a comparatively high antigen concentration for stimulation.

Lymph node cell cultures obtained from guinea pigs immunized with 3000 μg of DNP-GPA 7–17 days earlier also exhibited a characteristic pattern of responsiveness. A relatively high concentration of antigen, 10^{-2} μg/ml, was required to obtain significant stimulation of DNA synthesis. A considerable increment in ^{3}H-TdR incorporation was caused by increasing the concentration of DNP-GPA from 10^0 to 10^{+2} μg/ml. The response observed at 10^{+2} μg/ml was approximately 4 times that of unstimulated cultures. Furthermore, the relatively large increment in DNA synthesis noted as a consequence of increasing concentration of antigen from 10^0 to 10^{+2} μg/ml suggests that still higher antigen concentrations might have caused a further increase in thymidine incor-

poration. Thus, the estimated concentration of antigen (10^0 μg/ml) required for "50% stimulation" of thymidine uptake probably represents a minimum value. Nevertheless, it is clear that the response to immunization with a large dose of antigen is characterized by a requirement for relatively high antigen concen-

TABLE IV

Affinity of Purified Anti-DNP Antibodies Produced by Guinea Pigs Immunized with either 50 μg or 3000 μg of DNP-GPA

Immunizing dose of DNP-GPA.....	50 μg	3000 μg
Time after immunization	$\Delta F^{\circ *}$ for ε-DNP-L-lysine kcal/mole	
days		
7–9		−7.25‡
		−7.3‡
10	−8.04	−6.99
	−8.06	−7.08
	−7.89	
12	−8.87	
13	−8.90	−7.77
14		−7.25
		−7.55
		−7.68
15	−9.56	
17	−9.69	−7.35
	−9.62	−7.85
		−8.16
19	−9.75	

* $\Delta F^\circ = -R\,T \ln K_0$ where ΔF° is standard free energy of interaction, R the gas constant, T the absolute temperature, and K_0 the average intrinsic association constant.

‡ Purified antibody prepared from a pool of antisera; all other antibodies purified from serum of a single guinea pig.

tration to obtain stimulation of DNA synthesis. It appears as if the antigen-sensitive cell population, present at the time of culture, interacted with antigen, on the average, with relatively low affinity.

In order to rule out the possibility that antigen retained as a consequence of primary immunization artifactually influenced these results by its presence in the culture fluid, measurements of ^{3}H-DNP-GPA in culture fluid derived from guinea pigs immunized with radioactive antigen were made. Individual guinea

pigs were immunized with Freund's adjuvant emulsions containing either 1, 50, or 1360 μg of ^{3}H-DNP$_{28}$GPA (69 μc/mg of DNP-GPA). Cultures were prepared at various times after immunization and the amount of radioactivity in culture fluids measured. No antigen was detectable in culture fluid 7 days after immunization with 1 μg of ^{3}H-DNP-GPA (Table III). 7 days after immunization with 50 μg of ^{3}H-DNP-GPA, a barely detectable concentration of DNP-GPA was found (2×10^{-4} μg/ml), while no antigen was detected on day 11. 10 days after immunization with 1360 μg of ^{3}H-DNP-GPA $4\text{–}8 \times 10^{-4}$ μg/ml was found. Thus, in measurements of in vitro ^{3}H-TdR stimulation, contamination with antigen from the initial immunization does not appear to play any significant role with the possible exception of cultures derived from guinea pigs

TABLE V

^{3}H-TdR Incorporation into DNA in Cultures Derived From Guinea Pigs Immunized 5–7 Months Prior to Establishment of Culture

Concentration of DNP-GPA in culture fluid	Relative ^{3}H-TdR incorporation	
	Mean	Range
μg/ml		
10^{-4}	1.19*	(0.87–2.18)
10^{-3}	2.37	(1.24–4.91)
10^{-2}	3.24	(1.17–6.30)
10^{0}	4.29	(1.54–8.69)
10^{+2}	5.85	(2.31–10.18)

* Values reported are mean and ranges as obtained from cultures derived from individual guinea pigs immunized 5–7 months earlier with 50 μg of DNP-GPA.

Results are expressed on a scale relative to ^{3}H-TdR incorporation into DNA in cultures, from the same guinea pig, incubated in the absence of antigen. ^{3}H-TdR incorporation in such cultures was assigned a value of 1.00.

immunized with 3000 μg of DNP-GPA where responses to very low concentrations of DNP-GPA might have been obscured. However, in that event, control cultures derived from such guinea pigs would be expected to have exhibited a higher level of ^{3}H-TdR incorporation than control cultures obtained from animals immunized with either 50 or 1 μg. This was not observed, suggesting that even in this case the presence of antigen remaining from the immunizing injection did not significantly affect the results obtained, and that these cells could not react to these low antigen concentrations.

Affinity of Antibodies Produced Shortly after Immunization.—In order to relate the apparent binding characteristics of sensitive cell populations, present as a result of immunization with varying amounts of antigen, to the changes in the binding properties of the serum antibodies arising as a result of these immunization schedules, the binding affinity of the serum anti-DNP antibodies produced by such animals was measured. In general, guinea pigs immunized with 1 μg

of DNP-GPA did not produce sufficient anti-DNP antibodies shortly after immunization to permit purification and thus no measurements were made following this dose of antigen. Anti-DNP antibodies produced by guinea pigs at varying times from 10 to 19 days after immunization with either 50 or 3000 μg were specifically purified and hapten-binding affinity calculated from fluores-

TABLE VI

Affinity of Purified Anti-DNP Antibodies Produced at Various Times after Immunization with 50 μg of DNP-GPA

Time after immunization	ΔF° for DNPOH kcal/mole
10 days	−6.80
	−6.50
	−6.42
12 "	−6.43
13 "	−6.85
15 "	−7.68
17 "	−7.68
	−7.33
19 "	−7.55
5–7 months	−8.10
	−9.91
	−9.46
	−10.80
	−9.03

cence quenching titrations with ε-DNP-L-lysine. The affinity of anti-DNP antibodies produced by guinea pigs immunized with 50 μg of DNP-GPA was higher than that of anti-DNP antibodies produced by animals immunized with 3000 μg of DNP-GPA at all times between 10 and 19 days after immunization (Table IV). These findings correlate well with the requirement for greater antigen concentrations to obtain stimulation of cultures derived from guinea pigs immunized with 3000 μg of DNP-GPA as compared with cultures obtained from guinea pigs immunized with 50 μg of DNP-GPA.

In addition, a marked increase in the affinity of anti-DNP antibody is noted between 10 and 19 days after immunization with 50 μg of DNP-GPA. In contrast, the change in affinity seen in the antibodies produced by guinea pigs immunized with 3000 μg over the same time period is considerably less.

DNA Synthesis in Cultures Derived from Guinea Pigs Immunized with Antigens Other Than DNP-GPA.—The sensitivity of stimulation of in vitro DNA synthesis described here is considerably greater than that reported by previous workers (11, 12, 19, 20). In general, previous authors have noted that no significant stimulation of DNA synthesis was obtained with antigen concentrations of 10^0 μg/ml or less. Thus, the level of sensitivity observed in the current

TABLE VII

In Vitro ^{3}H-TdR Incorporation in Response to DNP-LiH and DNP-OVA in Cultures Derived From Guinea Pigs Immunized with DNP-GPA

Immunizing antigen	DNP-GPA		DNP-GPA		DNP-GPA	
Dose	50 μg		50 μg		3000 μg	
Duration before culture	5–7 months		7–15 days		7–8 days	
Test antigen	DNPLiH 100 μg/ml	DNPOVA 100 μg/ml	DNPLiH 100 μg/ml	LiH 100 μg/ml	DNPLiH 100 μg/ml	DNPLiH 1μg/ml
Incorporation* of ^{3}H-TdR relative to control cultures	2.60	2.16	2.11	0.88	1.90	0.91
	3.50	1.76	1.46	0.97	1.57	
	1.90	0.72	1.81		2.43	
	2.07		1.66			
	1.83					
Mean	2.38‡	1.55	1.76‡	0.93	1.97‡	0.91
SD	0.69		0.28		0.43	

* Results are expressed on a scale relative to ^{3}H-TdR incorporation into DNA in cultures, from the same guinea pig, incubated in the absence of antigen. ^{3}H-TdR incorporation in such cultures was assigned a value of 1.00.

‡ The mean values for relative DNP-LiH–stimulated ^{3}H-TdR incorporation in these cases differ significantly from the mean value obtained in cultures derived from guinea pigs immunized with Freund's adjuvant alone (Table II) with P values of <0.01.

experiments is as much as three to four orders of magnitude greater than that obtained by most previous workers. In order to demonstrate that this sensitivity was not peculiar to the DNP-GPA system, cultures were established with lymph node cells from guinea pigs immunized with 50 μg of either ovalbumin, DNP-BSA, or DNP-BGG. The sensitivity and concentration-responsiveness of such cultures were quite similar to those reported here for the DNP-GPA system. Thus, the level of sensitivity described in these experiments is by no means restricted to a single antigenic system.

Concentration Responsiveness of Cultures Established at Different Times after Immunization.—One very striking feature of the immune response is the in-

creasing affinity of the serum antibody with time after immunization. To study the behavior of cellular immunity as related to changes in antibody affinity, cultures were established from guinea pigs immunized with 50 μg of DNP-GPA 5–7 months earlier and the concentration-responsiveness of these cultures was compared with that of cultures established from similar guinea pigs 7–19 days after immunization. The results of individual cultures from nine guinea pigs at 5–7 months after primary immunization demonstrated no increase in sensitivity

TABLE VIII

Concentration of DNP-GPA Required to Give ^{3}H-TdR Incorporation Similar to that caused by 100 μg/ml DNPLiH in Cultures from DNP-GPA–Immunized Guinea Pigs

Immunizing dose					
50 μg		50 μg		3000 μg	
Duration before culture					
5–7 months		7–15 days		7–8 days	
DNPLiH relative incorporation*	Dose of DNP-GPA for equivalent response	DNPLiH relative incorporation	Dose of DNP-GPA for equivalent response	DNPLiH relative incorporation	Dose of DNP-GPA for equivalent response
	μg/ml		μg/ml		μg/ml
2.60	10^{-2}	2.11	$>10^{-4}, <10^{-2}$	1.90	10^{-2}
3.50	n.d.‡	1.46	$<10^{-4}$	1.57	10^{0}
1.90	$<10^{-2}$	1.81	$<10^{-2}$	2.43	$>10^{-2}, <10^{0}$
2.07	$>10^{-4}, <10^{-3}$	1.66	$<10^{-2}$		
1.83	$<10^{-4}$				

* Results are expressed on a scale relative to ^{3}H-TdR incorporation into DNA in cultures, from the same guinea pig, incubated in the absence of antigen. ^{3}H-TdR incorporation in such cultures was assigned a value of 1.00.

‡ n.d. = not done.

as compared with cultures derived from guinea pigs 7–19 days after immunization. There was no consistent difference between cultures established early and late in the course of immunization either with respect to minimum antigen concentration for significant stimulation of DNA synthesis or in terms of concentration required for "50% stimulation". However, these "late" cultures did display a great deal more variability in responsiveness than did cultures established relatively shortly after immunization. Table V presents average values and ranges for ^{3}H-TdR incorporation in these cultures.

In order to demonstrate that an increase in affinity of serum anti-DNP antibody with increasing time after immunization did, indeed, occur in this antigenic system, affinity measurements of purified serum anti-DNP antibodies produced early and late in the course of immunization with 50 μg of DNP-GPA

were made. Table VI shows that affinity of serum antibody for DNP-OH increased markedly between 10–19 days after immunization and 5–7 months after immunization. DNP-OH was used as ligand because ϵ-DNP-L-lysine is bound so tightly by 5–7 month antibody that accurate measurement by fluorescence quenching is not feasible. The magnitude of the increase in affinity with time, in terms of standard free energy of reaction (ΔF^0), is approximately -2 kcal/mole. The increase in affinity of serum antibody is of a sufficiently large degree so that if a comparably large increase in avidity of the antibody bound to the cells

TABLE IX

Effect of ϵ-DNP-L-Lysine on DNP-GPA Stimulation of In Vitro ^{3}H-TdR Incorporation

Immunizing and stimulating antigen	Antigen concentration in culture	ϵ-DPN-L-lysine concentration	Relative* ^{3}H-TdR incorporation
	μg/ml	μg/ml	
DNP-GPA	10^{-2}	0	3.07
		100	1.89
DNP-GPA	10^{-2}	0	2.60
		100	1.89
Ovalbumin	10^{-2}	0	2.49
		100	2.05
Ovalbumin	10^{-2}	0	2.95
		100	1.95

* Results are expressed on a scale relative to ^{3}H-TdR incorporation into DNA in cultures, from the same guinea pig, incubated in the absence of antigen. ^{3}H-TdR incorporation in such cultures was assigned a value of 1.00.

participating in antigen stimulated in vitro DNA synthesis had occurred, we would have expected to be able to detect this change with our methods.

In Vitro DNA Synthesis in Response to DNP-Protein Conjugates Different from That Used for Immunization.—The capacity of comparatively large amounts of DNP-OVA and DNP-GEL to elicit in vivo delayed responses in guinea pigs immunized with DNP-GPA was discussed above (Table I). It seemed likely that in vitro studies of this "cross-stimulation" phenomenon might allow more precise quantitation. The ability of DNP-LiH to stimulate DNA synthesis in lymph node cultures from DNP-GPA–immunized guinea pigs was studied in detail. In addition, the ability of DNP-OVA to cause such "cross-stimulation" was measured in a limited number of cultures.

In experiments described above (Table II), it was shown that DNP-LiH and DNP-OVA at concentrations of 10^{+2} μg/ml caused no measureable stimulation of DNA synthesis in cultures derived from guinea pigs immunized with Freund's adjuvant alone. Cultures derived from guinea pigs immunized with 50 μg of

DNP-GPA 5–7 months earlier were regularly stimulated to increased DNA synthesis by DNP-LiH at a concentration of 10^{+2} μg/ml. The mean level of incorporation of ^{3}H-TdR was 2.38 times that of unstimulated cultures. The concentration of the immunizing antigen, DNP-GPA, required to give a response equivalent to 10^{+2} μg/ml of DNP-LiH ranged from $<10^{-4}$ to 10^{-2} μg/ml (Table VIII). In two of three such cultures, DNP-OVA at 10^{+2} μg/ml caused significant stimulation (Table VII). Cultures derived from guinea pigs immunized with 50 μg of DNP-GPA 7–19 days earlier were stimulated by DNP-LiH (10^{+2} μg/ml) to incorporate ^{3}H-TdR at a level 1.76 times that of unstimulated cultures. This response is equivalent to that observed when such cultures are exposed to $<10^{-4}$–$<10^{-2}$ μg/ml of DNP-GPA. Cultures derived from guinea pigs immunized with 3000 μg of DNP-GPA 7–19 days earlier responded to 10^{+2} μg/ml of DNP-LiH with ^{3}H-TdR incorporation 1.97 times that of unstimulated cultures. These responses were equivalent to those obtained with concentrations of 10^{-2}–10^{0} μg/ml of DNP-GPA.

Thus, DNP-LiH and DNP-OVA caused significant stimulation of in vitro DNA synthesis. Under a variety of conditions, DNP-LiH was from 10^2 to $>10^6$ times less effective than DNP-GPA in eliciting responses in DNP-GPA–immunized animals. In addition, the data presented indicate that LiH alone caused no significant increase in DNA incorporation and that DNP-LiH at a concentration of 10^0 μg/ml (a concentration 100-fold less than that used in the experiments described above) failed to stimulate thymidine incorporation in cultures derived from DNP-GPA–sensitized animals (Table VII).

Hapten Inhibition of In Vitro Stimulation of DNA Synthesis.—ε-DNP-L-lysine at a concentration of 100 μg/ml (3.2×10^{-4}M) caused partial inhibition of the stimulation of DNA synthesis by 10^{-2} μg/ml of DNP-GPA in cultures obtained from guinea pigs immunized with 50 μg of DNP-GPA. In control experiments, this concentration of ε-DNP-L-lysine also had some depressive effect on in vitro stimulation of DNA synthesis by ovalbumin at 10^{-2} μg/ml in cultures derived from ovalbumin-immunized guinea pigs (Table IX). Concentrations of 10 μg/ml or less of ε-DNP-L-lysine exerted no measureable inhibitory effect in cultures derived from DNP-GPA–immunized guinea pigs. Thus, a concentration of ε-DNP-L-lysine 6×10^4 times greater than that of DNP in DNP-GPA was required for partial inhibition of DNA synthesis by 10^{-2} μg/ml of DNP-GPA. Furthermore, this inhibition could not be shown to be completely specific.

DISCUSSION

A quantitative approach to the study of the specificity of cellular immunity has been presented utilizing as a model system the in vitro antigen-stimulated increase in DNA synthesis of lymphoid cultures from immunized guinea pigs. The response of such cell cultures is markedly heterogeneous in the sense that graded responses occur over an exceedingly wide range of antigen concentrations

(10^{-4}–10^{+2} μg/ml). This type of response is most consistent with the existence of a heterogeneous population of sensitive cells, each bearing a specific antigen receptor of a characteristic structure and binding affinity. By virtue of its properties such a specific receptor for antigen may be referred to as an antibody. Thus, cells bearing high affinity antibody may be triggered with comparatively low concentrations of antigen whereas cells bearing lower affinity antibody would require relatively high concentrations of antigen to be stimulated. This interpretation is supported by the characteristic antigen concentration-response curves obtained in cultures derived from guinea pigs immunized with varying amounts of antigen. Thus, immunization with 1 μg of DNP-GPA leads to a population of cells which is sensitive to low antigen concentrations and which contains no appreciable number of cells requiring more than 10^{0} μg/ml to be triggered. As a result of immunization with small amounts of antigen, local antigen concentrations in lymphoid tissue sufficient to favor only cells bearing high affinity antibody are achieved. On the other hand, immunization with 3000 μg of DNP-GPA results in a cell population which does not appreciably respond to very low concentrations of DNP-GPA but which is stimulated by high concentrations of antigen. Such cells were initially stimulated because relatively high antigen concentrations were achieved due to the large immunizing dose. The lack of an appreciable response to a low concentration of DNP-GPA in cultures derived from guinea pigs immunized with 3000 μg of DNP-GPA may be due to the lack of a proliferative advantage of "high affinity" cells which may be assumed to have originally constituted only a small fraction of the total cells capable of responding to DNP-GPA. Alternatively, induction of tolerance in these high affinity cells by high antigen concentration may have occurred. The possibility must be considered, however, that the small amount of retained antigen present in fluid of cultures derived from 3000 μg–immunized guinea pigs may have obscured responses to very low antigen concentrations; but the fact that control cultures, derived from such animals, to which no antigen was added did not show unusually high thymidine incorporation suggests that the low concentration of antigen remaining from the immunizing injection did not significantly influence the results and that no cells were present capable of responding to these low concentrations of antigen. The concentration-responsiveness of cultures derived from guinea pigs immunized with 50 μg of DNP-GPA was intermediate between those of cultures obtained from 1 μg and from 3000 μg animals as would be expected.

Although it has been assumed in this discussion that the cells synthesizing DNA are themselves the sensitive cells, it is possible that DNA synthesis is a response of nonsensitized cells to some unknown mediator released by sensitive cells upon interaction with antigen. However, this possibility is still consistent with the thesis developed above that individual sensitive cells bind antigen with a characteristic affinity. If such a mediator is involved one need merely postulate

that the amount released is a function of the antigen bound by sensitive cells.

The observation that a large immunizing dose leads to a cellular immunity characterized by a low avidity type response in culture is completely consistent with the affinity of the serum antibody produced as a result of such an immunization schedule, as has been shown here and in previous studies (10).

These experiments are also consistent with the view that sensitized cells capable of responding to the immunizing antigen in vitro, and which are believed to be lymphocytes, are a heterogeneous population of cells with respect to their capacity to bind antigen and that probably each of these cells produces a unique antibody with characteristic affinity as appears to be the case for plasma cells (19, 20).

Both delayed reactions and in vitro stimulation of DNA synthesis in lymph node cell cultures were best achieved with the immunizing antigen, DNP-GPA, in confirmation of previous studies (1, 2, 21, 22). However, delayed responses were regularly elicited in DNP-GPA–immunized animals with relatively large amounts (200 μg) of DNP-GEL and of DNP-OVA, especially at 2 wk after immunization. Similarly, DNP-LiH and DNP-OVA caused significant stimulation of in vitro DNA synthesis in lymph node cell cultures derived from guinea pigs immunized with DNP-GPA. This response is specific for the conjugates as it was not duplicated by an equal concentration of LiH. Furthermore the observed responses are probably not the maximum responses obtainable with this antigen. Since 10^{0} μg/ml of DNP-LiH gave no response while 10^{+2} μg/ml gave a significant response it seems probable that still further increase in DNP-LiH concentration would lead to a graded increase in response. Finally, as in previous work (21, 22), free hapten, in this case ϵ-DNP-L-lysine, caused partial inhibition of DNP-GPA stimulation of cultures derived from DNP-GPA–immunized guinea pigs. However relatively high concentrations of hapten were required and the inhibition could not be shown to be completely specific.

An appreciation of the importance of the carrier protein in the interaction responsible for the initiation of the events involved in cellular immunity may be obtained from a determination of the concentration of DNP-GPA required to give a response equivalent to 10^{+2} μg/ml of DNP-LiH. DNP-GPA varied from $>10^{6}$ to 10^{2} times as effective as DNP-LiH on a weight basis.

Attempts to study the question of the "avidity" of cellular immune responses as a function of time after immunization failed to yield unequivocal results. However, no increase in antigen sensitivity of cultures could be demonstrated late in immunization. On the other hand, a very clear increase in ΔF^{0} of serum anti-DNP antibody was observed late in immunization as compared to antibody produced early in immunization. No explanation can be proposed for this apparent lack of change with time of the population of sensitized cells.

The data presented here provide additional support for the concept that antigen-cell interactions proceed on a specific, thermodynamically driven basis

(7, 9, 23) and point to some general similarity of specificity of cell-associated antibody and serum antibody. However, the similarity of specificity does not allow any conclusions about the nature of the cells which participate in antibody synthesis, in the secondary response, and in delayed hypersensitivity reactions. Furthermore, the state of the antigen at the time of contact with cell-associated antibody and the mechanism of induction remain unspecified in this study. In conclusion, one may state that the thermodynamically driven interaction of antigen (or "processed" antigen) with cell-associated antibody is necessary but may not be sufficient for stimulation of the events leading to the variety of immune responses. It is clear from this and from previous studies that the selection of a population of cells mediating any given type of specific immunological reaction can, in large part, be explained by the energetics of the interaction of antibody bound to these cells with some form of antigen.

SUMMARY

In vitro antigen stimulation of DNA synthesis in lymph node cultures from immunized guinea pigs can be obtained with very low (10^{-4} μg/ml) antigen concentrations in the culture fluid. Immunization with low doses of DNP-GPA leads to a cell population capable of being stimulated, on the average, by low concentration of antigen whereas immunization with large antigen doses results in a sensitive cell population requiring, on the average, high antigen concentrations for stimulation. These findings correlate well with the affinity for hapten of the serum antibodies produced by these guinea pigs.

Both delayed reactions in vivo and DNA synthesis in vitro can be stimulated by hapten conjugated to proteins different from that used in primary immunization. However the immunizing conjugate is much more effective in terms of antigen concentration required for a given response.

These results can be understood in terms of a thermodynamically driven interaction of antigen (or "processed" antigen) with cell-associated antibody.

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BIBLIOGRAPHY

1. Gell, P. G. H., and B. Benacerraf. 1961. Studies on hypersensitivity. IV. The relationship between contact and delayed sensitivity: A study of the specificity of cellular immune reactions. *J. Exptl. Med.* **113**:571.
2. Benacerraf, B., and B. B. Levine. 1962. Immunological specificity of delayed and immediate hypersensitivity reactions. *J. Exptl. Med.* **115**:1023.
3. Benacerraf, B., and P. G. H. Gell. 1959. Studies on hypersensitivity. III. The relation between delayed reactivity to the picryl group of conjugates and contact sensitivity. *Immunology.* **2**:219.
4. Ovary, Z., and B. Benacerraf. 1963. Immunological specificity of the secondary response with dinitrophenylated proteins. *Proc. Soc. Exptl. Biol. Med.* **114**:72.
5. Eisen, H. N. 1964–65. The immune response to a simple antigenic determinant. *Harvey Lectures.* **60**:1.

6. Rittenberg, M. B. 1966. Anamnestic anti-hapten response induced with hapten-heterologous protein conjugates. *Federation Proc.* **25**:548.
7. Paul, W. E., G. W. Siskind, B. Benacerraf, and Z. Ovary. 1967. Secondary antibody responses in haptenic systems. Cell population selection by antigen. *J. Immunol.* **99**: 760.
8. St. Rose, J. E. M., and B. Cinader. 1967. The effect of tolerance on the specificity of the antibody response and on immunogenicity. *J. Exptl. Med.* **125**:1031.
9. Paul, W. E., G. W. Siskind, and B. Benacerraf. 1967. A study of the "termination" of tolerance to BSA with DNP-BSA in rabbits: Relative affinities of the antibodies for the immunizing and the paralyzing antigens. *Immunology.* **13**:147.
10. Eisen, H. N., and G. W. Siskind. 1964. Variations in affinities of antibodies during the immune response. *Biochemistry.* **3**:996.
11. Dutton, R. W., and J. D. Eady. 1964. An *in vitro* system for the study of the mechanism of antigenic stimulation in the secondary response. *Immunology.* **7**:40.
12. Mills, J. A. 1966. The immunologic significance of antigen induced lymphocyte transformation *in vitro*. *J. Immunol.* **97**:239.
13. Carpenter, R. R. 1963. *In vitro* studies of cellular hypersensitivity. I. Specific inhibition of migration of cells from adjuvant-immunized animals by purified protein derivative and other protein antigens. *J. Immunol.* **91**:803.
14. David, J. R., S. Al-Askari, H. S. Lawrence, and L. Thomas. 1964. Delayed hypersensitivity *in vitro*. I. The specificity of inhibition of cell migration by antigens. *J. Immunol.* **93**:264.
15. Bray, C. A. 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. *Anal. Biochem.* **1**:279.
16. Reich, E., R. M. Franklin, A. J. Shatkin, and E. L. Tatum. 1962. Action of actinomycin D on animal cells and viruses. *Proc. Natl. Acad. Sci. U.S.* **48**:1238.
17. Farah, F. S., M. Kern, and H. N. Eisen. 1960. The preparation and some properties of purified antibody specific for the 2,4-dinitrophenyl group. *J. Exptl. Med.* **112**:1195.
18. Siskind, G. W., W. E. Paul, and B. Benacerraf. 1966. Studies on the effect of the carrier molecule on antihapten antibody synthesis. I. Effect of carrier on the nature of the antibody synthesized. *J. Exptl. Med.* **123**:673.
19. Nossal, G. J. V. 1962. Cellular genetics of immune responses. *Advan. Immunol.* **2**:163.
20. Green, I., P. Vassalli, V. Nussenzweig, and B. Benacerraf. 1967. Specificity of the antibodies produced by single cells following immunization with antigens bearing two types of antigenic determinants. *J. Exptl. Med.* **125**:511.
21. Dutton, R. W., and H. N. Bulman. 1964. The significance of the protein carrier in the stimulation of DNA synthesis by hapten-protein conjugates in the secondary response. *Immunology.* **7**:54.
22. Oppenheim, J. J., R. A. Wolstencroft, and P. G. H. Gell. 1967. Delayed hypersensitivity in the guinea pig to a protein-hapten conjugate and its relationship to *in vitro* transformation of lymph node, spleen, thymus and peripheral blood lymphocytes. *Immunology.* **12**:89.
23. Nussenzweig, V., and B. Benacerraf. 1967. Antihapten antibody specificity and L chain type. *J. Exptl. Med.* **126**:727.