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Hapten Specificity of Cellular Immune Responses as Compared with the Specificity of Serum Anti-hapten Antibody

W. E. PAUL AND G. W. SISKIND

Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014, *and Department of Medicine, Cornell University College of Medicine, New York, New York* 10021

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Summary. Comparative specificity data have been presented for the interaction of anti-hapten antibody with a series of structurally related haptens and for the stimulation by hapten-guinea pig albumin (GPA) conjugates of DNA synthesis in lymph node cell cultures from guinea pigs immunized with a given conjugate.

A good correlation in the specificity of serum anti-hapten antibody and in the specificity of stimulation of DNA synthesis has been demonstrated. Thus, mutual serologic and stimulatory cross-reactivity exist between the aminocaproates and GPA conjugates of the p-iodophenysulfonyl and toluenesulfonyl groups. Poor or undetectable serologic and stimulatory cross reactivity exist in the other combinations tested.

The data is consistent with the notion that the interaction of antigen with an antibody-like cellular receptor is crucial to the elicitation of cellular immune responses.

INTRODUCTION

One of the key features of the immune response is the capacity of a limited number of cells to respond in a characteristic way to a specific antigen. This cellular selection has been widely interpreted as indicating that immunocompetent cells have specific antigen recognition mechanisms possibly associated with their surface (see Siskind and Benacerraf, 1969). Furthermore, data obtained from the study of the elicitation of secondary immune responses strongly suggest that the cellular antigen recognition mechanism has a specificity very similar (or identical) to the specificity of the antibody molecules which are to be secreted by that cell or its progeny (Paul, Siskind, Benacerraf and Ovary, 1967; Mitchison, 1967).

The study of the specificity of cellular immune reactions and of the nature of the recognition mechanisms involved in such reactions has been more difficult than has been the case for the anamnestic antibody response because no specific product of the stimulated cells has been obtained. Previous studies with hapten conjugated proteins by Benacerraf and Levine (1962) and by Gell and Silverstein (1962) have attested to the importance of the effect of the carrier protein used for immunization upon the subsequent elicitation of delayed skin hypersensitivity. This readily observable phenomenon of carrier specificity has tended to obscure other aspects of the specificity of these reactions. Silverstein and

Gell (1962) have shown that structurally related haptens conjugated to the immunizing carrier protein elicit weaker reactions than does the immunizing hapten-protein conjugate. Thus the relevant cells appear to have the capacity to recognize haptenic groupings.

The introduction of *in vitro* models for cellular immune responses has allowed quantitative studies of the specificity of such responses. The antigen mediated stimulation of DNA synthesis in lymph node cell cultures from immunized animals (Dutton and Eady, 1964) has been particularly useful in this regard. We have recently studied the DNA synthetic response in culture utilizing a wide range of antigen concentrations for stimulation. An immunization schedule which results in serum anti-hapten antibody of high affinity also leads to cell populations which are sensitive to very low antigen concentrations and which thus appear to interact with antigen with high affinity. On the other hand, an immunization schedule leading to low affinity serum anti-hapten antibody also leads to cell populations requiring relatively high antigen concentrations for stimulation of DNA synthesis. Such cell populations appear to interact with antigen with relatively low affinity (Paul, Siskind and Benacerraf, 1968). The *in vitro* stimulation of DNA synthesis thus seems particularly well suited to the analysis of the specificity of the receptors involved in cellular immune responses.

In the current studies, we demonstrate that the capacity of related haptenic groupings, as hapten-GPA conjugates, to stimulate DNA synthesis *in vitro* in lymph node cells obtained from guinea pigs immunized with a given hapten-GPA conjugate is correlated with the degree of cross reactivity of serum anti-hapten antibody for these related haptens. The exquisite specificity for haptenic groupings and the pattern of cross reactivities provide inferential evidence for the similarity of the antigen receptors (recognition sites) of cells involved in cellular immune phenomena to serum type antibody.

MATERIALS AND METHODS

1-Fluoro-2, 4-dinitrobenzene (DNFB), 1-chloro-2, 4-dinitrobenzene (DNCB), toluenesulfonyl chloride (tosyl Cl), and para iodophenysulfonyl chloride (pipsyl Cl) were obtained from Eastman Organic Chemicals, Rochester, N.Y. Pentachlorobenzoyl chloride (Penta Cl) was obtained from E. I. Dupont de Nemours, Wilmington, Delaware. ε-Amino caproic acid (EACA) was purchased from Mann Research Laboratories, N.Y. EACA-α-ε-H^3 (EACA-H^3) was obtained from Tracerlab, Waltham, Massachussetts, and had a specific activity of 64 mcl/mM. Guinea pig albumin was prepared by electrophoresis on a polyvinyl chloride block (Geon) (Müller-Eberhard and Kunkel, 1956).

DNP-EACA-H^3, pipsyl-EACA-H^3, tosyl-EACA-H^3 and penta-EACA-H^3 were prepared by the reaction of EACA-H^3 with DNFB, pipsyl-Cl, tosyl-Cl and penta-Cl, respectively, in alkaline conditions at a 3 fold molar excess of DNFB or acid chloride in respect to EACA-H^3. The reaction was allowed to proceed overnight at room temperature. The products were purified by thin layer chromatography on silica gel using n-butanol. The purified compounds had RF's indistinguishable from the respective non-radioactive aminocaproates, which had been prepared essentially as previously described (Benacerraf and Levine, 1962).

Pipsyl-GPA, tosyl-GPA and penta-GPA were prepared by the reaction of 80 mg pipsyl-Cl, 40 mg tosyl-Cl and 40 mg penta Cl, respectively, with 200 mg GPA in alkaline conditions. In addition, a series of DNP-GPA conjugates were prepared, essentially as previously described (Benacerraf and Levine, 1962), using either DNFB or DNCB.

The degree of substitution of the DNP-GPA preparations was determined from absorbancy at 360 mμ (ε = 17,400 for ε-DNP-L-lysine, Carsten and Eisen, 1953) and Kjeldahl nitrogen measurements (molecular weight of GPA taken as 69,000). The degree of substitution of the uncoloured pipsyl-GPA, tosyl-GPA and penta-GPA was estimated by determining the difference in the number of DNP groups which could be introduced into an aliquot of previously conjugated GPA and the number of DNP groups which could be introduced into previously unconjugated GPA. An excess of DNFB was used for these reactions. This difference in the number of introduced DNP groups was taken as the degree of substitution obtained in the initial coupling step. Using these methods, pipsyl-GPA contained 23 moles hapten/mole GPA; tosyl-GPA 30 and penta-GPA 14. DNP-GPA preparations contained 10·5, 13·8, 22 and 40·1 moles DNP per mole of GPA ($DNP_{10\cdot5}$ GPA, $DNP_{13\cdot8}$GPA, DNP_{22}GPA and $DNP_{40\cdot1}$GPA).

Hartley strain guinea pigs, 300–400 g in weight, were immunized with Freund's complete adjuvant emulsions of hapten-GPA. Each guinea pig received a total dose of 50 μg of hapten-GPA injected into the four footpads. The adjuvant contained 2·0 mg of killed *M. tuberculosis* H37 Ra (Difco) per ml of oil phase. Two to four weeks later, the guinea pigs were bled; lymph nodes were removed under sterile conditions and cell cultures were established as previously described (Paul *et al.*, 1968) with two to four culture tubes at each point. Antigen was added immediately. Twenty-four hours later 1μc of thymidine[^{3}H]methyl was added. The cultures were terminated 24 hours later. The cells were washed twice in chilled phosphate buffered saline (PBS: 0·15 M NaCl, 0·01 M phosphate, pH 7·6); cold 0·5 M perchloric acid was then added. Thirty minutes later the precipitate was collected by centrifugation at 2000 RPM, 4° for 30 minutes. After 2 washes in cold 0·25 M perchloric acid and 1 in cold methanol, the precipitate was dried and dissolved in Hyamine. An aliquot was added to a scintillation mixture consisting of 25 parts toluene and 1 part Liquiflor (New England Nuclear Corp., Boston, Massachusetts). Radioactivity was measured in a liquid scintillation spectrometer. Degree of stimulation was determined by the following formula:

$$\frac{\text{Radioactivity in cultures to which antigen was added}}{\text{Radioactivity in cultures to which no antigen was added}} - 1.$$

Values in excess of 0 indicate stimulation of DNA synthesis by antigen. In these experiments, maximum degrees of stimulation observed averaged 6·8 and ranged from 2·9 to 14·3. In order to compare data from different experiments the maximum stimulation obtained in a given experiment was assigned a value of 1. All other stimulations are reported as their ratio to this reference stimulation (relative stimulation). Means of these normalized values were calculated and plotted.

Quantitative precipitin analyses of the antisera obtained were performed as previously described, employing hapten-bovine fibrinogen conjugates (Paul and Benacerraf, 1965). In each case, the hapten-fibrinogen conjugate contained the hapten which had been used in immunization. Average intrinsic association constants (K_0) were calculated from data obtained utilizing an ammonium sulfate precipitation technique (Stupp, Yoshida and Paul, 1969). Antisera (0·1 ml) either undiluted or diluted with an equal volume of PBS was mixed with 0·1 ml of hapten-EACA-H^3 at varying concentrations. After incubation at 4° for at least 30 minutes, two volumes of 66 per cent cold saturated ammonium sulfate were added. After an additional 30 minute incubation at 4°, the tubes were centrifuged at 2000 rev/min, 4° for 30 minutes and an aliquot of supernatant was transferred to 10 ml

of scintillation solvent containing 25 parts of toluene, 5 parts Bio-Solv 3 (Beckman Instrument Co., Fullerton, California) and 1 part Liquiflor, and radioactivity measured. This technique of estimating K_0 yields values somewhat lower than does equilibrium dialysis. This is particularly true when undiluted serum is used and when the ligand is an aminocaproate, presumably because of the binding of such ligands by albumin. Nevertheless, the data obtained can be utilized quite satisfactorily for the comparison of binding characteristics.

In those cases in which little or no direct binding of antiserum to a potentially cross reactive radioactive hapten was obtained, the capacity of high concentrations of that hapten, in the non-radioactive form, to inhibit the interaction of antiserum and the immunizing radioactive hapten was measured. The free concentration of the non-radioactive ligand required to inhibit 50 per cent of the initial binding of the radioactive ligand was determined. The ratio of this value to the concentration of non-radioactive immunizing hapten required for 50 per cent inhibition is inversely related to the ratio of the equilibrium constants of these ligands for antibody. Thus, an estimate of K_0 for poorly binding haptens could sometimes be obtained. In many instances, however, little or no inhibition was noted even at concentrations of 10^{-3}M. All such experiments were carried out under conditions in which at least 50 per cent of antigen-binding sites on antibody molecules had bound the radioactive reference ligand in the absence of inhibiting hapten.

Standard free energies of interaction ($\Delta F°$) were obtained from the relation:

$$\Delta F° = -RT \ln K_0$$

where R is the gas constant and T, the absolute temperature.

RESULTS

A. DNA synthetic response

In order properly to interpret data concerning the capacity of different hapten-GPA conjugates to stimulate DNA synthesis by lymph node cells it was first necessary to compare the relative stimulatory capacity of several preparations of a single hapten-GPA conjugate. Guinea pigs were immunized with either $DNP_{10\cdot5}GPA$ or $DNP_{13\cdot8}GPA$ and cultures were established 2–3 weeks later. The capacity of $DNP_{10\cdot5}GPA$, $DNP_{13\cdot8}GPA$ and $DNP_{40\cdot1}GPA$ to stimulate DNA synthesis in lymph node cells from these animals was determined in four independent experiments. $DNP_{13\cdot8}GPA$ and $DNP_{40\cdot1}GPA$ were, in general, similar in their stimulatory capacity with $DNP_{13\cdot8}GPA$ yielding slightly higher relative stimulation. $DNP_{10\cdot5}GPA$, although definitely stimulatory, was less effective than either of the other preparations (Fig. 1). This pattern of stimulation was obtained in cultures of cells from guinea pigs immunized with either $DNP_{10\cdot5}GPA$ or $DNP_{13\cdot8}GPA$. Thus, small differences in stimulatory capacity of various hapten-GPA conjugates may be due to factors other than the relative specificity of an individual receptor for two different ligands.

Studies of the DNA synthetic responses to pipsyl-GPA, tosyl-GPA, penta-GPA or $DNP_{22}GPA$ of lymph node cells obtained from animals immunized with a given hapten-GPA conjugate revealed a high degree of specificity. In each case, the immunizing hapten-GPA complex was the most efficient stimulant. Fig. 2 presents responses for lymph node cells obtained from guinea pigs which had been immunized with one of the four hapten-

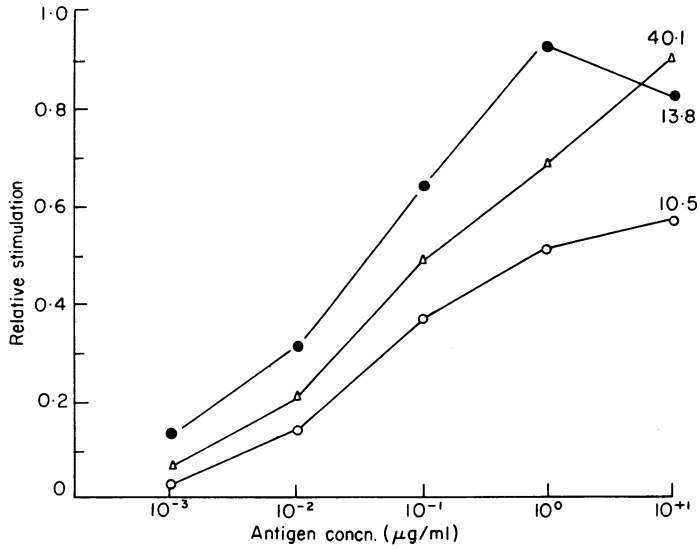

FIG. 1. Effect of degree of substitution on the capacity of DNP-GPA conjugates to stimulate DNA synthesis in lymph node cell cultures obtained from DNP-GPA immunized guinea pigs. The data presented are the means of relative stimulation obtained in 4 independent experiments, 2 performed on cells from guinea pigs immunized with $DNP_{10\cdot5}$ GPA and 2 performed on cells from guinea pigs immunized with $DNP_{13\cdot8}$ GPA. The number next to curves are the degrees of substitution (moles DNP/mole GPA) of the preparations used for stimulation. Antigen concentration refers to concentration used in culture for stimulation.

GPA complexes. These data are the mean responses of 4 experiments each for cells derived from DNP_{22}GPA, pipsyl GPA and tosyl GPA immunized animals. Only a single experiment was performed on cells obtained from a penta-GPA immunized animal. Each experiment utilized cells from a single animal and different animals were used for each experiment.

In brief, cells from guinea pigs immunized with tosyl-GPA could be stimulated both by tosyl and by pipsyl-GPA and cells from guinea pigs immunized with pipsyl-GPA could be stimulated both by pipsyl-GPA and by tosyl-GPA. Cells from guinea pigs immunized with either DNP-GPA or penta-GPA were appreciably stimulated only by the immunizing conjugate.

A more detailed presentation of the data summarized above is as follows. Clear cross stimulation was observed in cultures obtained from animals immunized with pipsyl-GPA (Fig. 2b) and with tosyl-GPA (Fig. 2c). In these cases the immunizing antigen stimulated very well and penta-GPA and DNP_{22}GPA did not stimulate at all. Tosyl-GPA, at a concentration of 10^2 μg/ml, stimulated cells from pipsyl-GPA immunized animals almost as well as did pipsyl-GPA. Pipsyl-GPA, at a concentration of 10^2 μg/ml, stimulated cells from tosyl-GPA immunized animals equally as well as did tosyl-GPA. In both instances, the immunizing antigen was a superior stimulant at 10^{-2} and 10^0 μg/ml.

Cells from DNP_{22}GPA immunized guinea pigs (Fig. 2a) were stimulated maximally with 10^0 μg DNP_{22}GPA/ml and significantly with 10^{-2} μg/ml. No measurable stimulation was caused by pipsyl, tosyl or penta-GPA in any concentration tested. In the single experiment on cells from a penta-GPA immunized guinea pig (Fig. 2d), penta-GPA stimulated measurably at 10^{-2} μg/ml and maximally at 10^2 μg/ml. DNP_{22} GPA and pipsyl-GPA stimulated marginally at 10^{-2} to 10^2 μg/ml.

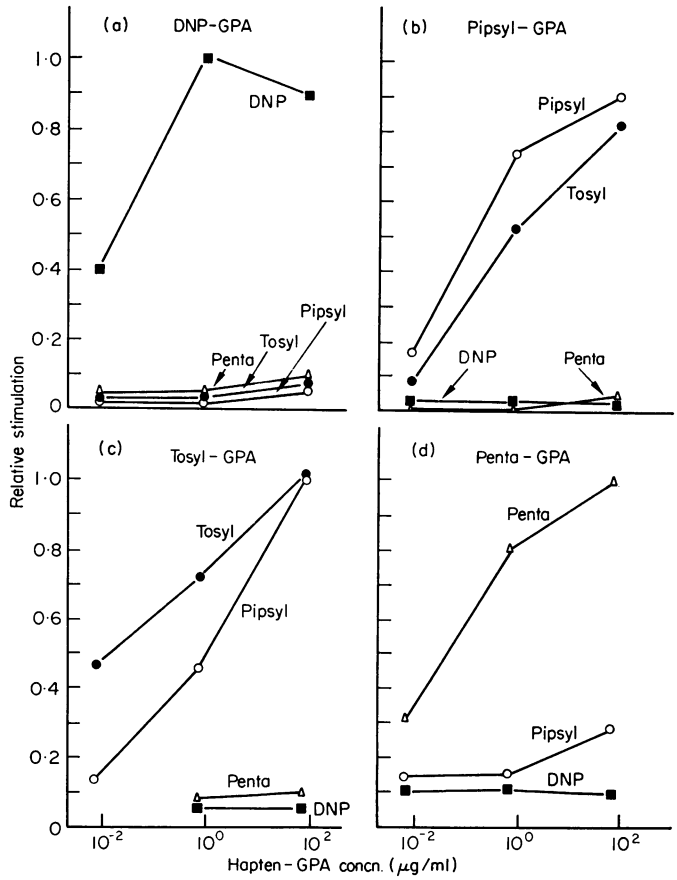

FIG. 2. Relative stimulation of DNA synthesis in lymph node cell cultures by a series of related hapten-GPA conjugates. The data presented are the means of relative stimulation for four independent experiments on cells from animals immunized with $DNP_{22}GPA$ (2a), pipsyl-GPA (2b) and tosyl-GPA (2c). A single experiment is depicted in 2d on cells from a penta-GPA immunized guinea pig. The hapten shown next to a given curve represents the hapten-GPA conjugate used to obtain that stimulation curve.

B. HAPTEN BINDING CHARACTERISTICS

The study of the hapten binding characteristics of antisera obtained from animals immunized with either DNP-GPA, pipsyl-GPA, tosyl-GPA or penta-GPA yielded results quite consonant with the stimulatory data presented in Section A. Thus, a high degree of cross reaction was noted between pipsyl-EACA and tosyl-EACA both for anti-pipsyl and anti-tosyl antisera and only weak or undetectable cross reactions were noted for other combinations.

Table 1 presents detailed results of binding of anti-hapten antisera by hapten-EACA conjugates. Antisera to tosyl-GPA bound both tosyl-EACA-H^3 and pipsyl-EACA-H^3 very well. In direct binding experiments DNP-EACA-H^3 were bound very poorly by anti-tosyl antibody (Fig. 3). Inhibition studies on one antiserum (tosyl-1) showed that DNP-EACA was bound with approximately 2·5 kcal/mole less energy than was tosyl-EACA and with 2·4 kcal/mole less energy than was pipsyl-EACA. Penta-EACA caused no measurable inhibition of binding of tosyl-EACA-H^3.

TABLE 1

BINDING OF ANTI-HAPTEN-GPA ANTIBODY TO RELATED LIGANDS

Serum	Ligand: Pipsyl-EACA	Tosyl-EACA	DNP-EACA	Penta-EACA
Anti-Pipsyl-GPA				
Pool	7·98*	7·07	≪5·5†	n.i.
2–21	7·45	7·25	≪4·7†	n.i.
1	6·57	5·29	n.b.	n.b.
2	7·53	6·52	n.b.	n.b.
Mean	7·38	6·53		
Anti-Tosyl-GPA				
Pool	7·08	7·42		
2–21x	7·85	7·81		
1	6·33	6·43	3·9‡	n.i.
2	6·55	6·20	n.b.	n.b.
Mean	6·95	6·97		
Anti-DNP-GPA				
1	n.i.	n.i.	6·84	≪4·7†
2	n.i.	n.i.	7·32	≪4·5†
3	n.i.	n.i.	7·78	≪5·7†
4	n.i.	n.i.	7·99	≪5·8†
Mean			7·49	
Anti-Penta-GPA				
5	n.i.	≪5·5†	5·7‡	7·37

* Standard free energy of interaction ($-\Delta F°$), in kcal/mole, of the interaction of ligand with antibody.

† Binding estimated by inhibition of reaction of antiserum and homologous radioactive ligand. Less than 50 per cent inhibition occurred at the highest concentration tested. The cited value is the calculated $-\Delta F°$ had 50 per cent inhibition been achieved at highest tested ligand concentration. In each of these cases extrapolation of the obtained inhibition curve indicates that the actual $-\Delta F°$ is much lower than the value given.

‡ Determined by inhibition as above. In these instances 50 per cent inhibition was achieved at highest concentration tested.

n.i.—No measurable inhibition of reaction of antiserum and homologous radioactive hapten achieved at highest concentration tested.

n.b.—No measurable direct binding of radioactive ligand to antiserum.

Anti-pipsyl-GPA antisera bound pipsyl-EACA-H^3 with a mean standard free energy of interaction ($\Delta F°$) of $-7·38$ kcal/mole. Tosyl-EACA-H^3 was bound with, on the average, 0·85 kcal/mole less energy than was pipsyl-EACA-H^3. In direct binding studies, neither DNP-EACA-H^3 nor penta-EACA-H^3 was measurably bound. Inhibition studies showed some inhibition (but less than 50 per cent) with the highest concentration of DNP-EACA tested (10^{-3}M) and no inhibition with that concentration of penta-EACA.

Anti-DNP-GPA sera exhibited excellent direct binding with ^{3}H-DNP-EACA (mean $\Delta F° = -7·49$ kcal/mole). No measurable inhibition was obtained with either pipsyl or tosyl-EACA at 10^{-3}M. Penta-EACA caused some (but less than 50 per cent) inhibition at 10^{-3}M.

Finally, with the single anti-penta-GPA serum tested, penta-EACA-H^3 was bound with a $\Delta F° -7·37$ kcal/mole. Inhibition tests revealed that DNP-EACA was bound with approximately 1·7 kcal/mole less energy than was penta-EACA. Tosyl-EACA gave less than 50 per cent inhibition at 10^{-3}M and pipsyl-EACA, no inhibition.

Thus, animals immunized with pipsyl-GPA produced antibodies which bound both pipsyl-EACA and tosyl-EACA quite well and their lymph node cells could be significantly stimulated both with pipsyl-GPA and with tosyl-GPA. Similarly, sera from animals immunized with tosyl-GPA bound tosyl-EACA and pipsyl-EACA almost equally well and cells from these animals were stimulated with either tosyl-GPA or pipsyl-GPA. Other combinations showed minimal or absent cellular cross stimulation and weak or absent antibody cross reactivity.

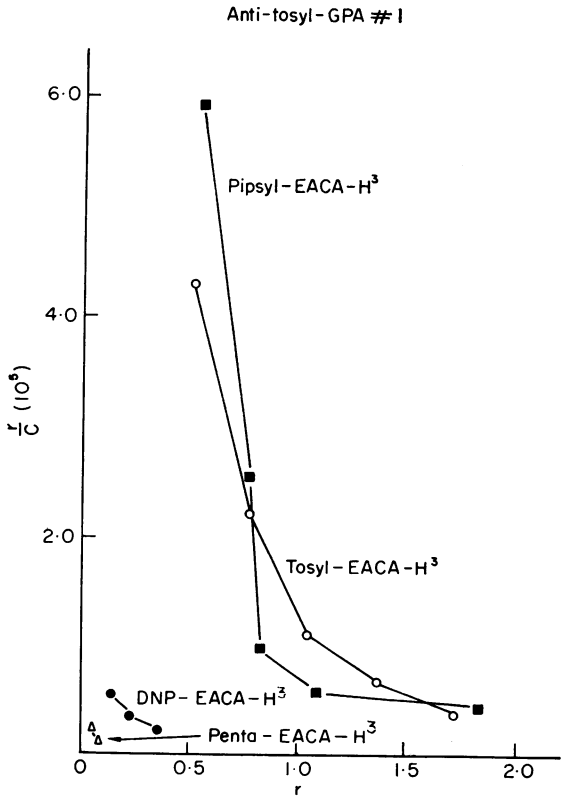

FIG. 3. Equilibrium binding curves of guinea pig anti tosyl-GPA antibody with tosyl-EACA-H^3, pipsyl-EACA-H^3, DNP-EACA-H^3 and penta-EACA-H^3. The data are plotted according to the expression $r/c = nk - rk$, where r is the concentration of hapten bound per concentration of antibody molecules, c is the free concentration of hapten, n is the valance of antibody and k is the equilibrium constant.

DISCUSSION

In the current study, a relation has been demonstrated between the capacity of a given hapten-GPA conjugate to stimulate DNA synthesis in lymph node cells obtained from immunized animals and the relative capacity of serum from such animals to bind that hapten-EACA conjugate. In those cases in which a high degree of serological (binding) cross reactivity existed, a significant stimulatory cross reactivity was also present. When binding cross reactivity of serum antibody was poor or not measurable, no stimulatory cross reactivity could be demonstrated. The cellular immune response appeared to be somewhat more discriminatory than was antibody. However, this might well be anticipated both from the experiment, reported here, in which several DNP-GPA preparations were used to stimulate cells from DNP-GPA immunized animals and from the following considerations. (1) Cell stimulation in culture involves the interaction of an antigen molecule with multiple similar determinants with a cell bearing multiple identical receptors sites. A significant opportunity exists for the formation of multiple bonds between an individual cell and one antigen molecule which should serve to magnify the small energetic differences observed in the binding of univalent ligands by antibody (Paul, Siskind and

Benacerraf, 1966; Hornick and Karush, 1969). (2) Some determinants are probably generated as a result of the covalent interaction of hapten and GPA (Haurowitz, 1942; Eisen, Carsten and Belman, 1954; Paul, Siskind and Benacerraf, 1967). These are represented on the immunizing hapten-GPA conjugate and may serve to stimulate a group of cells to synthesize DNA. The absence of these determinants both from other hapten-GPA conjugates and from all hapten-EACA conjugates would thus add a discriminatory capacity to the cellular reactions not present in the anti-hapten antibody. It should be noted that this would not imply any basic difference in type of specificity. (3) Conformation of hapten groups as GPA conjugates and as aminocaproates may not be identical and may thus influence apparent relative specificity. (4) Interactions *in vivo* very likely do not occur under equilibrium conditions. Thus the lack of precise concordance between data obtained in the equilibrium binding of hapten by antibody *in vitro* and in the antigen stimulation of cells is not surprising.

It thus appears that cellular immune responses, such as the stimulation of DNA synthetic responses in lymph node cell cultures, have specificity characteristics similar to those of antibody. These studies, then, support the concept that an interaction of antigen with an antibody-like receptor is a crucial step in the initiation of cellular immune responses. These receptors are very likely associated with the surface membranes of lymphocytes as suggested from studies of Wigzell and Andersson (1969), Naor and Sulitzeanu (1969), Ada and Byrt (1969) and Humphrey and Keller (1969). However this interaction of receptor and antigen, although necessary, may not be sufficient for the stimulation of specific cells.

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