ANTIGEN RECOGNITION: IN VITRO STUDIES ON THE SPECIFICITY OF THE CELLULAR IMMUNE RESPONSE*

By STUART F. SCHLOSSMAN,‡ M.D., JUDITH HERMAN, M.D., and ARIEH YARON, Ph.D.

(From the Department of Medicine, Harvard Medical School, Beth Israel Hospital, Boston, Massachusetts 02215, and the Department of Biophysics, The Weizmann Institute of Science, Rehovoth, Israel)

(Received for publication 19 May 1969)

While it is clear that cellular immune responses are characterized by a high degree of specificity, the mechanism by which the immunologically committed cell recognizes antigen is poorly understood. The specificity of antigen recognition has been determined from immunochemical studies of the antigens necessary to elicit various forms of cellular immunity. These studies have indicated that the recognition of antigen by an immunologically committed cell involves the participation of an exquisitely specific receptor system which can discriminate between closely related antigens (1–5). In the DNP-L-lysine system,[1] the same chemical characteristics of the antigen are required to elicit or desensitize to the delayed skin reaction, to provoke the anamnestic response, and to elicit cellular immune reactions in vitro (as measured by incorporation of thymidine-2-^{14}C or production of macrophage-inhibiting factor) (6–10). In contrast, nonimmunogenic DNP-oligolysines (containing fewer than 7 L-lysyl residues) and DNP-substituted proteins, all of which react with preformed anti-α-DNP-oligolysine antibody to provoke immediate hypersensitivity reactions in vivo or antigen-antibody reactions in vitro, are incapable of triggering the α-DNP-oligolysine–sensitized cell. These studies suggest the presence of a binding site on the sensitized lymphoid or processing cell which differs from the binding site of humoral anti-hapten antibody. The chemical nature of this binding site or its distinction from a unique form of immunoglobulin, however, has not been resolved.

Exploration of the nature of the receptor requires a chemically defined antigen system and a highly sensitive measure of the cellular immune response. The recently developed in vitro models of cellular immunity have provided

* This work was supported by National Science Foundation grant GB-6675K, U.S. Public Health Service grant AI 09003, and agreement 234103 with the National Institutes of Health.

‡ Recipient of career development award K03 HE11666 from the U.S. Public Health Service.

[1] *Abbreviations used in paper*: DNP, 2,4-dinitrophenyl; TCM, tissue culture medium.

such a measure (11–14). In the experiments reported below, the specificity of antigen-induced thymidine-2-^{14}C incorporation into sensitized lymph node cells was investigated. Lymph node cells were obtained for culture from inbred strain 2 guinea pigs sensitized with α-DNP-oligolysines, ϵ-DNP-oligolysines, oligolysines and α-DNP-oligolysines with D-lysine substituents. Cell cultures were exposed to both the homologous immunizing antigen and closely related derivatives of these antigens; the specificity of the cellular receptor for antigen was estimated from the chemical requirements of the antigen necessary to induce thymidine-2-^{14}C incorporation.

Materials and Methods

α-N-DNP-oligolysine Peptides.—α-*N*-DNP-pentalysine (α-DNP-Lys$_5$) and α-*N*-DNP-nonalysine (α-DNP-Lys$_9$), containing a DNP group on the single N-terminal α-amino position of the oligo-L-lysine chain (all lysine derivatives are of the L configuration unless otherwise indicated), were prepared by ion exchange chromatography from α-*N*-DNP-oligolysines of low average molecular weight as previously described (7, 15). α-*N*-DNP-nonalysines with single predetermined D-lysine substituents of the nonalysine chain were prepared by the stepwise synthesis procedure of Merrifield (16, 17). The preparation of α-*N*-DNP-nonalysine [α-DNP-Lys$_9$ (L$_4$DL$_4$)] containing a D-lysine substituent at the fifth lysyl residue of the nonalysine chain was described in an earlier publication (18). Two additional α-*N*-DNP-nonalysines containing single D-lysine substituents at the second and eighth lysyl residues, respectively, were prepared by the stepwise synthesis procedure and are designated α-DNP-Lys$_9$ (LDL$_7$) and α-DNP-Lys$_9$ (L$_7$DL).

ϵ-N-DNP-oligolysines.—A homologous series of oligolysine peptides containing a single dinitrophenyl substitution on the ϵ-amino group of the C-terminal lysine was prepared by Yeda, Rehovoth, Israel, by polymerization of ϵ-*N*-benzyloxycarbonyl-α-*N*-carboxyl-L-lysine anhydride, using ϵ-*N*-DNP-L-lysine benzyl ester as the initiator of polymerization. The benzyloxycarbonyl and benzyl ester groups were cleaved with 30% HBr in acetic acid for 1 hr at room temperature and for 72 hr at 4°C. The latter mixture of peptides, Lys$_{\overline{13}}$·ϵ-DNP-Lys, was designated Lys$_{\overline{14}}$·ϵ-DNP. The nonalysine member, Lys$_8$·ϵ-DNP-Lys, of this homologous series of peptides was previously described and designated 9-ϵ-DNP-Lys$_9$ (10). A nonalysine containing a single dinitrophenyl group on the ϵ-amino position of the fifth lysyl residue (Lys$_4$·ϵ-DNP-Lys·Lys$_4$) was prepared by the stepwise synthesis procedure and designated 5-ϵ-DNP-Lys$_9$.

Oligolysines.—Pentalysines (Lys$_5$), a mixture containing octa-, nona-, and decalysine (Lys$_{8\text{-}10}$), and a mixture containing tri-, tetra-, penta-, and hexalysine (Lys$_{3\text{-}6}$) were prepared from a hydrolysate of polylysines of high molecular weight as previously described (19, 6).

Proteins and Dinitrophenyl-Protein Conjugates.—Dinitrophenylated human serum albumin was described in an earlier publication (9), and tuberculin-purified protein derivative was purchased from Merck Sharp and Dohme (West Point, Pa.).

Spectrophotometry.—A Gilford spectrophotometer with silica cells of 1 cm light path was used for all spectrophotometric determinations. The α-DNP-lysine content of the peptides was determined in 0.1 M sodium phosphate–saline buffer, pH 7.0, at 3600 A ($E_{3600\,A}$ = 16,800). The ϵ-DNP-lysine content was similarly determined ($E_{3600\,A}$ = 17,400).

Immunization.—Guinea pigs of inbred strain 2 weighing approximately 400 g were immunized either with complete Freund's adjuvant (containing H37Ra tubercle bacilli, Difco Laboratories, Detroit, Mich.) or with 250 μg of the test antigen in complete Freund's adjuvant. 0.1 ml of the saline-adjuvant emulsion was injected into each of the four footpads.

Skin Tests.—11–14 days following immunization, intradermal skin tests were performed with 10–50 μg of the immunizing antigen in 0.1 ml of buffered saline solution. The test sites were observed at 3–6 hr and 24 hr, and the extent of induration and erythema was measured.

Preparation of Lymphocyte Cultures.—Approximately 3–4 days following skin tests, the animals were killed by cervical dislocation. The axillary, inguinal, and popliteal nodes were removed under sterile conditions and washed twice in minimum essential medium (Grand Island Biological Co., Grand Island, N.Y.). The nodes were trimmed of fat and teased with forceps into tissue culture medium (minimal essential medium containing 13% normal guinea pig serum; penicillin, 50 units/ml; streptomycin, 50 μg/ml; L-glutamine; and nonessential amino acids). Large tissue aggregates were permitted to settle, and the lymphocyte-containing supernatant was aspirated. The lymph node cells were harvested from the supernatant by centrifugation at 190 *g*, washed with TCM, and resuspended in 4.0 ml TCM. Living cells were counted in a hemocytometer by trypan blue exclusion. Additional TCM was added to provide a concentration of 2×10^7 viable cells/ml. Cell viability was consistently greater than 70%. 1 ml of TCM, 0.03–0.2 ml of the antigen to be tested, and 0.5 ml of the lymph node cells were added to each culture tube. Duplicate or triplicate tubes were prepared for each compound at each dose level in individual experiments. Occasionally, however, when many doses of the same antigen were used in a single experiment, single cultures were prepared at each dose. After 24 hr of incubation at 37°C, an additional 1.0 ml of TCM containing 0.2 μc of thymidine-2-^{14}C (New England Nuclear Corporation, Boston, Mass.) was added to each tube. After an additional 24 hr, the cultures were terminated by centrifugation, and the individual cell pellets were washed twice with Hanks' basic salt solution (Grand Island Biological Co., Grand Island, N.Y.), dissolved in NCS reagent (Nuclear-Chicago, Des Plaines, Ill.), and counted in a liquid scintillation counter. In each experiment the antigen-induced incorporation of thymidine-2-^{14}C was compared with the amount of thymidine-2-^{14}C incorporated into antigen-free cultures prepared from the same animal. The ratio of counts per minute in tubes containing antigen to the counts per minute of antigen-free tubes is referred to as the stimulation index. Each antigen was tested in cultures from at least three immunized animals. Results for a given dose level of each antigen in two or more identically sensitized animals were averaged for the sake of simplicity in the presentation of the data. Although the absolute values of the stimulation index obtained in different animals were variable, the composite curves presented reflect the pattern of results in each individual experiment.

RESULTS

In Vivo and In Vitro Response to DNP-oligolysines and Oligolysines.—Strain 2 guinea pigs were skin-tested with 10–50 μg of the homologous antigen 11–14 days following immunization. All animals immunized with α-DNP-Lys_9, 5-ε-DNP-Lys_9, or $Lys_{\overline{14}}$·ε-DNP developed delayed reactions to the 10 μg test dose, with lesions averaging 8, 10, and 25 mm in diameter, respectively. In contrast, none of the animals immunized with $Lys_{8\text{-}10}$, α-DNP-Lys_9(LDL$_7$), or α-DNP-Lys_9(L$_7$DL) responded to the 10 μg test dose. However, approximately 50% of the animals receiving α-DNP-Lys_9(LDL$_7$) or α-DNP-Lys_9(L$_7$DL) and 10% of animals treated with $Lys_{8\text{-}10}$ responded to 50 μg with a delayed reaction averaging 7 mm in diameter. Nonresponding animals were not retested, but their cells were used as immunized cells for studies of antigen-induced thymidine-2-^{14}C incorporation in vitro.

In every case tested, antigens which provoked an observable skin reaction also elicited an in vitro response. Of considerable interest, however, were the

observations that Lys_{8-10}, α-DNP-$Lys_9(LDL_7)$, and α-DNP-$Lys_9(L_7DL)$ elicited maximal stimulation indices of 15, 15, and 11, respectively, despite negative delayed skin reactions in more than half the animals challenged with 50 μg. No significant difference in the in vitro response was noted between cultures from animals which showed a delayed skin reaction and animals which did not. Athough it may be argued that repeated skin testing with larger amounts of antigen and more vigorous immunization would have led to consistently positive skin tests, these observations suggest that the in vitro response as measured by thymidine incorporation is either a more sensitive assay or, alternatively, not a simple in vitro correlate of delayed hypersensitivity. The response may, in fact, be a more basic manifestation of the general phenomenon of cellular immunity, which includes the proliferation of cells involved in delayed hypersensitivity, antibody production, and other, still undefined, immunological events.

To establish that antigen-induced thymidine-2-^{14}C incorporation is a specific consequence of prior immunization, the effect of antigen on thymidine incorporation was tested in cell cultures derived from animals immunized to adjuvant alone. In these cultures, as has been shown previously (9), the average maximal stimulation index produced by DNP-oligolysines and oligolysines in dosages between 1.8×10^{-9} and 5×10^{-6} M was 2.0. In contrast, in lymph node cell cultures derived from animals immunized with α-DNP-Lys_9, 5-ϵ-DNP-Lys_9, $Lys_{\overline{14}} \cdot \epsilon$-DNP, α-DNP-$Lys_9(LDL_7)$, α-DNP-$Lys_9(L_7DL)$, or Lys_{8-10}, the maximal stimulation indices were between 8.8 and 27 (Figs. 1–6).

As has been previously demonstrated, a minimum chain length of 7 L-lysine residues was required to elicit a cellular immune response. 16 guinea pigs were immunized with α-DNP-$Lys_9(L_4DL_4)$, α-DNP-Lys_5, Lys_{3-6}, and Lys_5. None of these animals developed an immune response; the homologous antigen neither provoked a delayed skin reaction nor induced the incorporation of thymidine-2-^{14}C in vitro. α-DNP-Lys_5, Lys_{3-6}, Lys_5, and α-DNP-$Lys_9(L_4DL_4)$ were tested at doses ranging from 1.8×10^{-9} to 5×10^{-6} M; the stimulation indices obtained were no different from those obtained with the identical antigens in lymph node cell cultures derived from animals immunized to adjuvant alone.

Dose-Response Curves for Homologous and Related Antigens.—The effect of the homologous immunizing antigen and of closely related antigens on thymidine-2-^{14}C incorporation in sensitized lymph node cell cultures is shown in Figs. 1–6. It is apparent that the immunizing antigen produced maximum stimulation on a molar basis. For example, in cultures obtained from six animals sensitized to $Lys_{\overline{14}} \cdot \epsilon$-DNP (Fig. 1), 1.8×10^{-8} M antigen produced a stimulation index of 27; with larger doses the stimulation index decreased. Doses of $Lys_{\overline{14}} \cdot \epsilon$-DNP larger than 1.8×10^{-8} M had a depressant effect on cell cultures derived from animals sensitized to and stimulated by DNP–human serum albumin and purified protein derivative. Similar toxic effects of large

doses of antigen have been reported with α-DNP-Lys_{11-15} and randomly substituted DNP-polylysines (9, 20). 9-ϵ-DNP-Lys_9, an immunogenic component of the $Lys_{\overline{14}}$·ϵ-DNP series of peptides, yielded stimulation indices indistinguishable from the mixture of $Lys_{\overline{14}}$·ϵ-DNP peptides. In contrast, 5-ϵ-DNP-

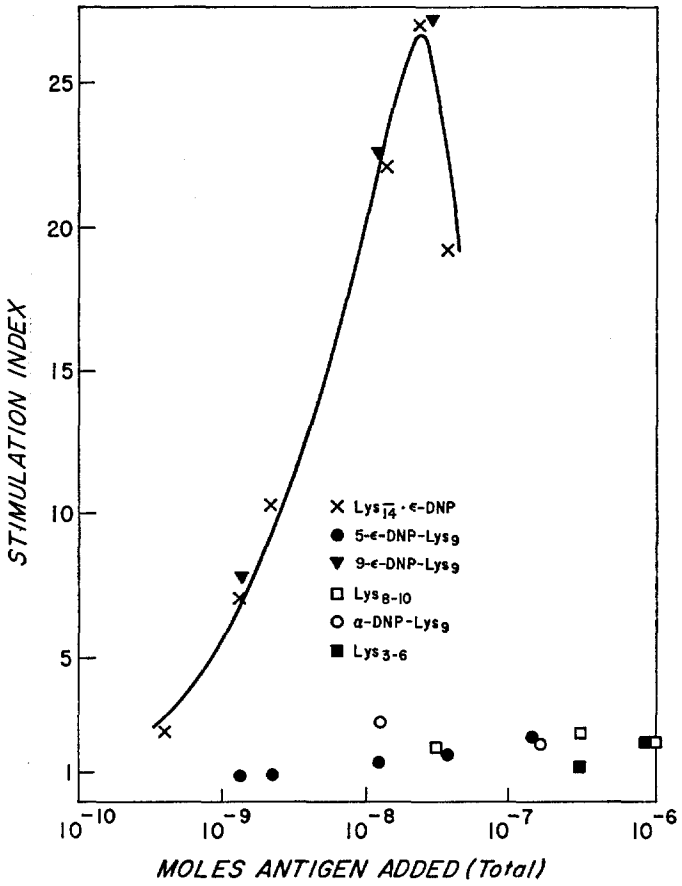

FIG. 1. Stimulation index provoked by varying quantities of DNP-oligolysines and oligolysines in lymph node cell cultures derived from six animals sensitized to $Lys_{\overline{14}}$·ϵ-DNP. The stimulation index is the ratio of antigen-induced thymidine-2-^{14}C incorporation to thymidine-2-^{14}C incorporation in antigen-free cell cultures.

Lys_9 containing the identical 9 L-lysyl residues, but with the DNP group on the ϵ-amino position of the fifth lysyl residue, was not stimulatory. 1.8×10^{-7} M 5-ϵ-DNP-Lys_9 gave a stimulation index of 2.2, whereas a 100-fold lower concentration (1.8×10^{-9} M) of $Lys_{\overline{14}}$·ϵ-DNP or 9-ϵ-DNP-Lys_9 produced stimulation indices of 7–8. Similarly, α-DNP-Lys_9 and Lys_{8-10}, despite their demon-

strated ability to stimulate their respective homologous cell cultures (Figs. 3 and 6), could not trigger cells from animals sensitized to $Lys_{\overline{14}}$·ε-DNP. In addition, no response was obtained with Lys_{3-6}.

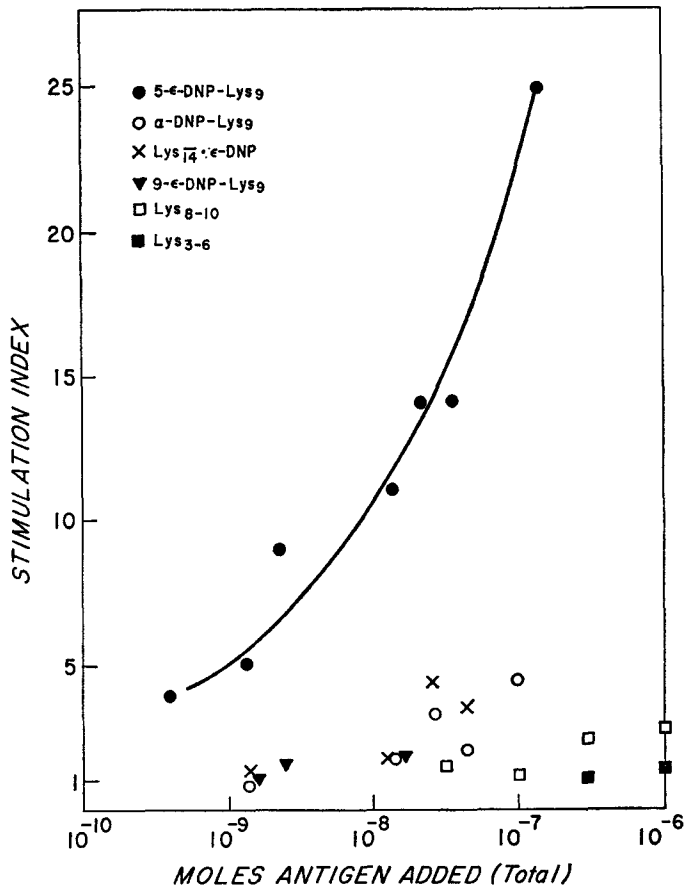

FIG. 2. Stimulation index provoked by varying quantities of DNP-oligolysines and oligolysines in lymph node cell cultures derived from five animals sensitized to 5-ε-DNP- Lys_9.

A further example of the specificity of the lymph node cells for antigen was obtained in five animals sensitized to 5-ε-DNP-Lys_9. As is shown in Fig. 2, 1.8×10^{-7} M immunizing antigen produced a stimulation index of 25. Closely related compounds containing the identical ε-DNP group on a different lysyl residue, oligolysines with the DNP group on the α-N-terminal amino position, and oligolysines without the DNP group failed to produce comparable stimulation. Thus, $Lys_{\overline{14}}$·ε-DNP, 9-ε-DNP-Lys_9, α-DNP-Lys_9, and Lys_{8-10} were

only minimally capable of provoking thymidine-2-^{14}C incorporation as compared to the homologous antigen.

Although related antigens were, in general, unable to yield stimulation indices comparable to the homologous immunizing compound at any given dose, significant cross-reactions were obtained in cell cultures derived from animals sensitized to α-DNP-Lys$_9$, α-DNP-Lys$_9$(LDL$_7$), and α-DNP-Lys$_9$(L$_7$DL). For example, in cultures derived from eight animals sensitized to α-DNP-Lys$_9$,

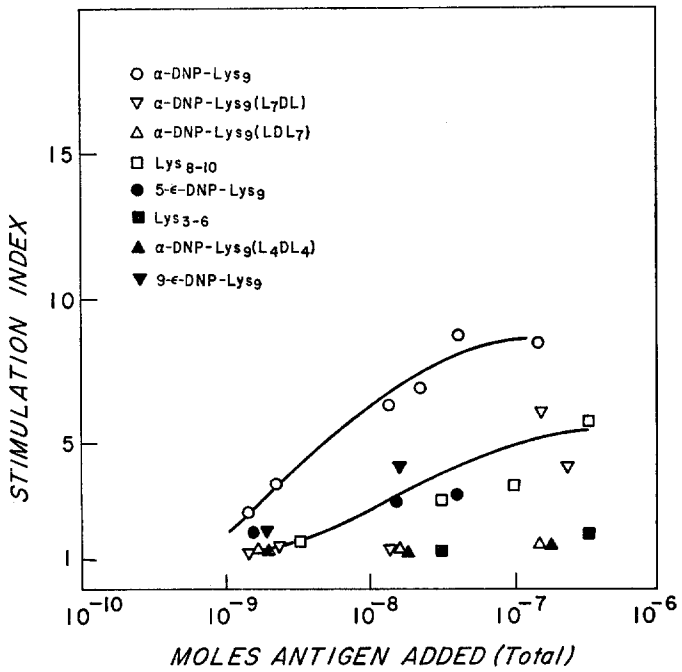

FIG. 3. Stimulation index provoked by varying quantities of DNP-oligolysines and oligolysines in lymph node cell cultures derived from eight animals sensitized to α-DNP-Lys$_9$.

the maximal stimulation index elicited by this antigen was 8.8 (Fig. 3). Cross-reactions were obtained with Lys$_{8\text{-}10}$, 5-ϵ-DNP-Lys$_9$, 9-ϵ-DNP-Lys$_9$, and α-DNP-Lys$_9$(L$_7$DL) (Fig. 3). No stimulation was noted with α-DNP-Lys$_9$(LDL$_7$), α-DNP-Lys$_9$(L$_4$DL$_4$), or Lys$_{3\text{-}6}$. The observation that there is little difference between the response to Lys$_{8\text{-}10}$ and the response to α-DNP-Lys$_9$(L$_7$DL), 5-ϵ-DNP-Lys$_9$, or 9-ϵ-DNP-Lys$_9$ suggests that the DNP group in a nonhomologous position did not strikingly improve the capacity of oligolysines to provoke thymidine-2-^{14}C incorporation (Fig. 3). Similar observations were made in lymph node cultures derived from animals sensitized to α-DNP-Lys$_9$(LDL$_7$) and α-DNP-Lys$_9$(L$_7$DL) (Figs. 4 and 5). The homologous immunizing antigens

were most effective on a molar basis in stimulating these cultures to incorporate thymidine-2-^{14}C, and the cultures were triggered by closely related antigens, e.g. α-DNP-Lys_9, 5-ϵ-DNP-Lys_9, α-DNP-$Lys_9(L_7DL)$, α-DNP-$Lys_9(LDL_7)$, and $Lys_{8\text{-}10}$. Again, $Lys_{8\text{-}10}$ without the DNP group was effective in provoking thymidine-2-^{14}C incorporation, whereas $Lys_{3\text{-}6}$ and α-DNP-$Lys_9(L_4DL_4)$ were not.

Since the results suggest that the oligolysine portion of the peptide plays an

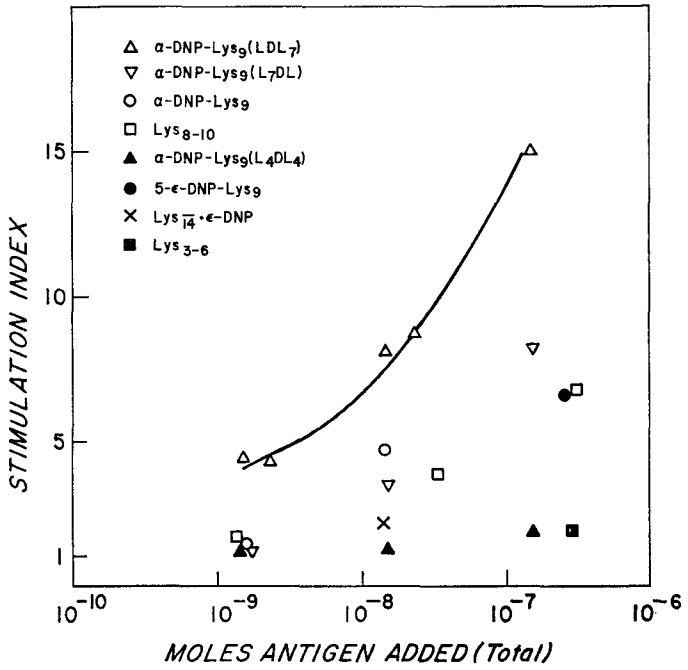

FIG. 4. Stimulation index provoked by varying quantities of DNP-oligolysines and oligolysines in lymph node cell cultures derived from four animals sensitized to α-DNP-$Lys_9(LDL_7)$.

important role in the specificity of cross-reactions, an experiment was performed to determine whether the hapten was essential for inducing a population of sensitized lymph node cells. 10 animals were immunized with $Lys_{8\text{-}10}$, and the cultures were shown to incorporate thymidine-2-^{14}C in response to $Lys_{8\text{-}10}$ (Fig. 6), although the concentration necessary to achieve a response was higher than in the cases of DNP-oligolysines. In cultures, $Lys_{8\text{-}10}$ and α-DNP-Lys_9 appear equally effective in provoking thymidine-2-^{14}C incorporation. Of the other compounds tested, $Lys_{\overline{14}}\cdot\epsilon$-DNP, 5-$\epsilon$-DNP-$Lys_9$, and α-DNP-$Lys_9(L_7DL)$ all produced cross-reactions. Since the animals sensitized to $Lys_{8\text{-}10}$ had no prior contact with the dinitrophenyl group, the ability of these cultures

to respond to DNP-oligolysines could only be ascribed to the oligolysine portion of the DNP-oligopeptide. Compounds without a sequence of 7 L-lysines, i.e. Lys_{3-6} and α-DNP-$Lys_9(L_4DL_4)$, were unable to trigger these cultures.

DISCUSSION

An immunochemical study of the specificity of the sensitized lymph node cell for antigen has been presented, utilizing the technique of antigen-induced

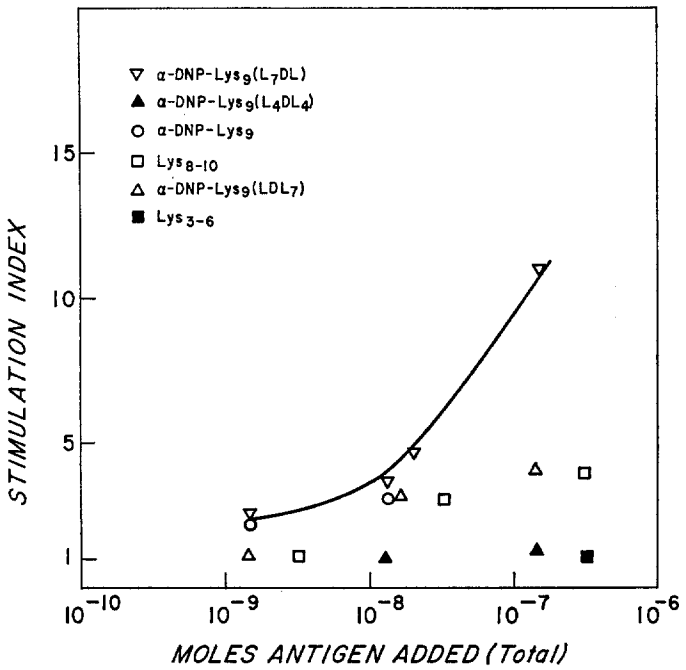

FIG. 5. Stimulation index provoked by varying quantities of DNP-oligolysines and oligolysines in lymph node cell cultures derived from four animals sensitized to α-DNP-$Lys_9(L_7DL)$.

thymidine-2-^{14}C incorporation in vitro. It is assumed that the antigen-sensitive lymph node cell is an immunologically committed small lymphocyte and that thymidine-2-^{14}C incorporation is an indirect measure of the interaction of antigen with a complementary and stereochemically defined receptor on such cells, an interaction which triggers cellular proliferation. Although the precise nature of the cells involved in antigen recognition are still undefined, the present studies indicate that the receptor exhibits an extraordinary degree of specificity for the immunizing antigen. In fact, the specificity of this response lends additional support for the thesis that the sequential and conformational determinants of

the immunogen are not degraded prior to the induction of the immune response, but are recognized in their entirety by the immunologically competent or processing cell (18, 21, 22). For example, lymph node cells obtained from inbred strain 2 guinea pigs immunized with a series of closely related antigens, i.e. α-DNP-Lys_9, α-DNP-$Lys_9(LDL_7)$, α-DNP-$Lys_9(L_7DL)$, $Lys_{\overline{14}}\cdot\epsilon$-DNP, 5-$\epsilon$-DNP-$Lys_9$, and $Lys_{8\text{-}10}$, can discriminate among these peptides and are maximally

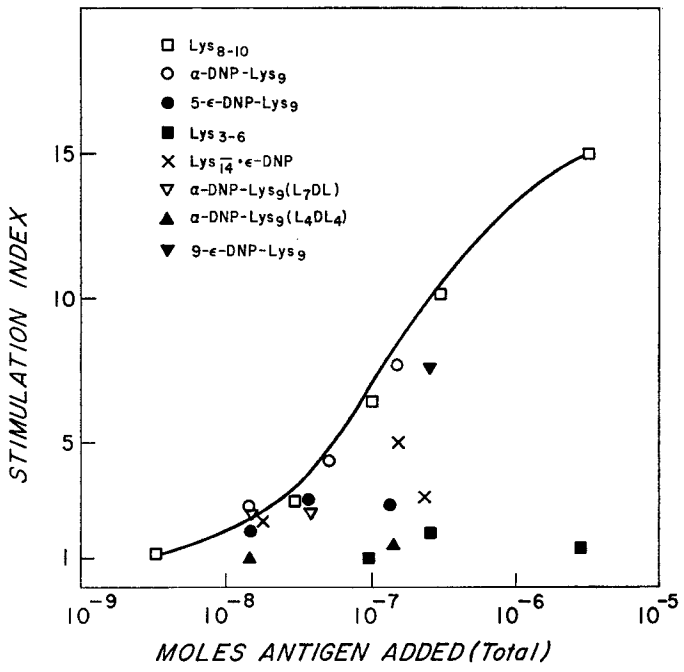

FIG. 6. Stimulation index provoked by varying quantities of DNP-oligolysines and oligolysines in lymph node cell cultures derived from 10 animals sensitized to $Lys_{8\text{-}10}$.

stimulated by the immunizing antigen to incorporate thymidine-2-^{14}C (Figs. 1–6). In cultures derived from animals immunized with $Lys\cdot_{\overline{14}}\epsilon$-DNP, only this compound and 9-ϵ-DNP-Lys_9 provoked significant stimulation (Fig. 1). Despite the similarity in lysine and hapten content and the demonstrated capacity of 5-ϵ-DNP-Lys_9, α-DNP-nonalysines, and $Lys_{8\text{-}10}$ to stimulate their respective cell cultures, virtually no stimulation was produced by these antigens in $Lys_{\overline{14}}\cdot\epsilon$-DNP cultures. Similarly, in cultures obtained from animals sensitized to 5-ϵ-DNP-Lys_9, closely related peptides provoked only minimal thymidine-2-^{14}C incorporation (Fig. 2). In contrast, cell cultures from animals immunized to α-DNP-Lys_9, α-DNP-$Lys_9(L_7DL)$, and α-DNP-$Lys_9(LDL_7)$ were readily induced to incorporate thymidine by closely related immunogenic DNP-oligoly-

sines and oligolysines (Figs. 3–5). How can one account both for the lack of cross-reactions in $Lys_{\overline{14}}$·ε-DNP and 5-ε-DNP-Lys_9 cell cultures and for the cross-reactions obtained in cultures from animals sensitized to α-DNP-Lys_9, α-DNP-Lys_9(LDL$_7$), and α-DNP-Lys_9(L$_7$DL)? One possibility is that at least two populations of specifically sensitized cells are produced as a consequence of immunization with DNP-oligolysines. Most of the cells have specific receptors for the DNP-oligolysine used to induce the response; a smaller and more variably sized population of cells is specific for the immunogenic oligolysine portion of the molecule. According to this hypothesis, the cells bearing the DNP-oligolysine receptor can be triggered only by the homologous immunizing antigen, whereas the oligolysine-specific cells, which acount for the cross-reactions, can be triggered by heterologous DNP-oligolysines. In support of this view, it was noted that in every instance when cells sensitized to α-DNP-Lys_9, α-DNP-Lys_9(L$_7$DL), or α-DNP-Lys_9(LDL$_7$) were stimulated by heterologous hapten-substituted antigens, $Lys_{8\text{-}10}$ was also stimulatory (Figs. 3–5). When cross-reactions were minimal or absent, i.e. in $Lys_{\overline{14}}$·ε-DNP and 5-ε-DNP-Lys_9 cultures, $Lys_{8\text{-}10}$ was not stimulatory (Figs. 1 and 2). The lack of an appreciable response to heterologous DNP-oligolysines without a corresponding response to $Lys_{8\text{-}10}$ suggests that the oligolysine peptide plays a crucial role in these cross-reactions. Further support for this view is found in cell cultures derived from animals sensitized to $Lys_{8\text{-}10}$ and stimulated by DNP-oligolysines (Fig. 6). Since these animals had no prior exposure to the DNP group, the stimulation indices must have resulted from the common oligolysine peptide. However, some DNP-oligolysines were less effective than $Lys_{8\text{-}10}$ in stimulating $Lys_{8\text{-}10}$-sensitized cells. These results suggest that the DNP group or D-lysine residue may sterically alter or prevent the interaction of the oligolysine portion of the molecule with the $Lys_{8\text{-}10}$-binding site. Accordingly, the failure to obtain cross-reacting cells in animals immunized with $Lys_{\overline{14}}$·ε-DNP or 5-ε-DNP-Lys_9 results from the ε-DNP group, which can either restrict sensitization to or alter the configuration of the oligolysine portion of the molecule. Under these circumstances, oligolysine-sensitive cells may be produced, but in numbers too small for detection by the in vitro system. More sensitive techniques or the use of larger numbers of cells for in vitro culture may be required to detect these cells. The present studies, however, provide no explanation as to why the ε-DNP group is more effective than the α-DNP group or D-lysine residue in preventing the establishment of a detectable population of oligolysine-sensitive cells.

Alternatively, one might postulate that immunization induces the formation of a homogeneous population of cells derived perhaps from a single clone and having identical receptors which can combine with the entire hapten-oligolysine complex as well as with portions of the determinant. Large doses of heterologous hapten-substituted peptides, nonimmunogenic α-DNP-oligolysines (9), and oligolysines should, we believe, under these circumstances, trigger a response

of the same order of magnitude as the complete immunizing antigen; within a 100-fold dose range this did not occur. While toxicity and limited supplies of antigen have prevented a systematic study of the effects of heterologous antigens and nonimmunogenic α-DNP-oligolysines at doses significantly higher than those tested in Figs. 1–6, it is believed that the data do not support a homogeneous cell hypothesis but favor the view that lymphoid cells are induced with receptors of extremely restricted specificity to each immunogenic determinant on the immunizing antigen.

Evidence obtained by Paul et al. (23) also indicated that a heterogeneous population of cells was formed as a consequence of immunization with hapten-protein conjugates. However, these authors concluded that the heterogeneity reflected a variation in affinity of cell-associated antibody for the haptenic group of the immunizing antigen. In the present studies, the demonstration that the oligolysine portion of the molecule, and not the haptenic group, plays a major role in determining the specificity of cross-reactions of cells strongly suggests that cell-associated antibody of the conventional type is not involved in cellular recognition of antigen. Numerous studies have shown that the major proportion of the binding energy of antibody formed to hapten-substituted peptides and proteins is directed at the immunodominant haptenic group; the peptide and protein carriers contribute only a small portion of the total binding energy (24, 25, 7). For example, α-DNP-Lys_{3-6} reacts with anti-α-DNP-Lys_9 antibody to provoke immediate hypersensitivity reactions in vivo or inhibition of precipitation of anti-α-DNP-Lys_9 antibody in vitro, whereas Lys_{8-10}, lacking the DNP group, will not do so (6, 7, 18). Nevertheless, Lys_{8-10}, but not α-DNP-Lys_{3-6} or α-DNP-$Lys_9(L_4DL_4)$, can induce α-DNP-Lys_9–sensitized cells to incorporate thymidine-2-^{14}C. Were the cross-reactions of cells solely dependent on the variation in the affinity of anti-hapten cell-associated antibody, α-DNP-Lys_{3-6} and α-DNP-$Lys_9(L_4DL_4)$, and not Lys_{8-10}, would have been expected to provoke substantial thymidine-2-^{14}C incorporation.

Although the studies reported above do not bear directly on the "cell cooperation hypothesis" suggested by Mitchison (26), we are reluctant to support the view that separate cells bearing hapten- and carrier-oriented receptors are involved in a cooperative act of antigen recognition prior to cell stimulation. Earlier studies have shown that the upper limit on binding site size on anti-α-DNP-Lys_9 antibody is complementary to α-DNP-Lys_7 (7). Similar studies with polysaccharide, polynucleotide, and polypeptide determinants for other antigen-antibody systems have given values comparable in size to the heptamer as the upper limit for combining site size (27). Under such circumstances, it remains a strong possibility that a single cellular receptor for antigen can encompass both the haptenic and "carrier portion" of the immunogenic molecule, and that the pronounced carrier effect noted in cellular immune reactions reflects the specificity of the cellular receptor for the total determinant used to induce the response.

The observation that α-DNP-Lys_9(L$_7$DL) and α-DNP-Lys_9(LDL$_7$), stereoisomers of nonimmunogenic α-DNP-Lys_9(L$_4$DL$_4$), can induce the immune response provides additional support for the view that the oligolysine backbone is crucial for immunogenicity, and that the hapten, its position, and the bond by which it is coupled to the oligolysine are less critical. This requirement for a sequence of 7 L-lysine residues to induce the immune response suggests that the initial step of immunogen recognition in the DNP-lysine system may involve an additional recognition system or processing event (perhaps macrophage-dependent) for the oligolysine portion of the immunizing antigen. This step of immunogen recognition appears to be under genetic control and inherited as a Mendelian dominant trait in the guinea pig (28–30). The subsequent step(s) of immunogen recognition, which presumably involves the selection and establishment of a population of sensitized cells with an exquisitely specific recognition system for the hapten-oligolysine and the capacity to produce antibody and other mediators involved in the immune response, is less clearly defined. A crucial and still unresolved point is whether the triggering of the established cellular immune response involves precisely the same steps of immunogen recognition as are involved in the initial induction of the immune response. Under any circumstances, however, it appears that as a consequence of immunization with a totally synthetic but closely related series of compounds varying only in the position of D-lysine residues and an identical hapten, animals of inbred strain 2 can elaborate populations of sensitized cells having receptors of different specificities for each of these compounds.

SUMMARY

Studies of the immunochemical specificity of antigen-induced thymidine-2-^{14}C incorporation in lymph node cells obtained from animals immunized to a series of closely related α-DNP-oligolysines, ϵ-DNP-oligolysines, and oligolysines have shown that the sensitized cell exhibits an extraordinary degree of specificity for antigen. The sensitized cell is maximally stimulated by the homologous immunizing antigen and can discriminate among compounds which differ from one another only in the position of a dinitrophenyl group or D-lysine residue on an identical oligolysine backbone. These studies support the view that the immunogen is not degraded prior to the induction of the immune response, and that the majority of cells produced as a consequence of immunization have stereospecific antigen receptors for the DNP-oligolysine used to induce the response; a smaller and more variably sized population of cells is produced with receptors specific for the oligolysine portion of the immunizing antigen. When specifically sensitized lymph node cell cultures are stimulated in vitro by heterologous DNP-oligolysines, the oligolysine– and not the DNP-oligolysine–sensitive population of cells appears to play a crucial role in the specificity of such cross-reactions. It is concluded from these studies that the antigen receptor on the sensitized lymph node cell differs in both kind and de-

gree from conventional antibody. The chemical nature of the receptor and the means by which this receptor reacts with antigen to initiate the biosynthetic or proliferative cellular immune response still remain undefined.

BIBLIOGRAPHY

1. Gell, P. G. H., and B. Benacerraf. 1961. Delayed hypersensitivity to simple protein antigens. *Advan. Immunol.* **1**:319.
2. Salvin, S. B., and R. F. Smith. 1960. The specificity of allergic reactions. *J. Exp. Med.* **111**:465.
3. Benacerraf, B., and B. B. Levine. 1962. Immunological specificity of delayed and immediate hypersensitivity reactions. *J. Exp. Med.* **115**:1023.
4. Gell, P. G. H., and A. M. Silverstein. 1962. Delayed hypersensitivity to hapten-protein conjugates. I. The effect of carrier protein and site of attachment to hapten. *J. Exp. Med.* **115**:1037.
5. Silverstein, A. M., and P. G. H. Gell. 1962. Delayed hypersensitivity to hapten-protein conjugates. II. Anti-hapten specificity and the heterogeneity of the delayed response. *J. Exp. Med.* **115**:1053.
6. Schlossman, S. F., S. Ben-Efraim, A. Yaron, and H. A. Sober. 1966. Immunochemical studies on the antigenic determinants required to elicit delayed and immediate hypersensitivity reactions. *J. Exp. Med.* **123**:1083.
7. Schlossman, S. F., and H. Levine. 1967. Immunochemical studies on delayed and Arthus-type hypersensitivity reactions. I. The relationship between antigenic determinant size and antibody combining site size. *J. Immunol.* **98**:211.
8. Schlossman, S. F., and H. Levine. 1967. Desensitization to delayed hypersensitivity reactions: with special reference to the requirement for an immunogenic molecule. *J. Immunol.* **99**:111.
9. Stulbarg, M., and S. F. Schlossman. 1968. The specificity of antigen-induced thymidine-2-^{14}C incorporation into lymph node cells from sensitized animals. *J. Immunol.* **101**:764.
10. David, J. R., and S. F. Schlossman. 1968. Immunochemical studies on the specificity of cellular hypersensitivity: the in vitro inhibition of peritoneal exudate cell migration by chemically defined antigens. *J. Exp. Med.* **128**:1451.
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. Schlossman, S. F., A. Yaron, S. Ben-Efraim, and H. A. Sober. 1965. Immunogenicity of a series of α, N-DNP-L-lysines. *Biochemistry (Washington).* **4**:1638.
16. Merrifield, R. B. 1964. Solid-phase peptide synthesis. 4. The synthesis of methionyl-lysyl-bradykinin. *J. Org. Chem.* **29**:3106.

17. Merrifield, R. B. 1964. Solid-phase peptide synthesis. 3. An improved synthesis of bradykinin. *Biochemistry*. **3**:1385.
18. Yaron, A., and S. F. Schlossman. 1968. Preparation and immunologic properties of stereospecific α, dinitrophenylnonalysines. *Biochemistry* (*Washington*). **7**:2673.
19. Yaron, A., A. Berger, E. Katchalski, M. C. Otey, and H. Sober. 1964. Preparation and properties of oligolysyl-peptides. *VI Int. Congr. Biochem.* **II**:190.
20. Green, I., W. E. Paul, and B. Benacerraf. 1968. Hapten carrier relationships in the DNP-PLL, foreign albumin complex system: induction of tolerance and stimulation of cells in vitro. *J. Exp. Med.* **127**:43.
21. Schlossman, S. F., H. Levine, and A. Yaron. 1968. Studies on the specificity of antibody to 2,4,dinitrophenyl-poly-L-lysines. *Biochemistry* (*Washington*). **7**:1.
22. Sela, M., B. Schechter, I. Schechter, and F. Borek. 1967. Antibodies to sequential and conformational determinants. *Cold Spring Harbor Symp. Quant. Biol.* **32**:537.
23. Paul, W. E., G. W. Siskind, and B. Benacerraf. 1968. 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. *J. Exp. Med.* **127**:25.
24. Eisen, H. N., and G. W. Siskind. 1964. Variations in affinities of antibodies during the immune response. *Biochemistry* (*Washington*). **3**:996.
25. Paul, W. E., G. W. Siskind, and B. Benacerraf. 1966. Studies on the effect of the carrier molecule on anti-hapten antibody synthesis. II. Carrier specificity of anti 2,4-dinitrophenyl-poly-L-lysine antibodies. *J. Exp. Med.* **123**:689.
26. Mitchison, N. A. 1967. Antigen recognition responsible for the induction in vitro of the secondary response. *Cold Spring Harbor Symp. Quant. Biol.* **32**:431.
27. Kabat, E. A. 1968. Structural Concepts in Immunology and Immunochemistry. Holt Rinehart and Winston, Inc., New York.
28. Levine, B. B., A. Ojeda, and B. Benacerraf. 1963. Studies on artificial antigens. III. The genetic control of the immune response to hapten·poly·L·lysine conjugates in guinea pigs. *J. Exp. Med.* **118**:453.
29. Levine, B. B., A. Ojeda, and B. Benacerraf. 1963. Basis for antigenicity of hapten poly-L-lysine conjugates in random bred guinea pigs. *Nature* (*London*). **200**:544.
30. Benacerraf, B , I. Green, and W. E. Paul. 1967. The immune response of guinea pigs to hapten·poly·L·lysine conjugates as an example of the genetic control of the recognition of antigenicity. *Cold Spring Harbor Symp. Quant. Biol.* **32**:569.