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ABSTRACT

The stimulus for this research, concerned with the role of the carrier in antibody formation, was the finding that polyanions such as nucleic acids could be converted into effective immunogens by coupling them to the basic protein methylated bovine serum albumin. This suggested the hypothesis that antibody formation is initiated by the reaction of antigen with mediator cells through carrier groups of the antigen.

This interaction has proved to be generally specific, requiring antibody-like receptors on the surface of the mediator cells. Non-specific charge interaction also contributes to the binding of antigen to mediator cell, thus accounting for the carrier activity of basic proteins. Although the receptor of mediator cells has not been defined, the evidence is that the mediator cells belong to a thymus-processed population of lymphocytes (T cells).

Such T cells have been isolated in a functional state from the spleen of mice very early in the course of their response to sheep red blood cells. These cells have antigen on their surface, are resistant to 1000 R of x-radiation, do not synthesize antibody but cooperate in the induction of antibody synthesis by antibody-producing B cells derived from bone marrow. These data provide direct evidence for the current dogma that antibody formation to thymus-dependent antigens requires the cooperation of at least 2 types of cells, T and B cells.

Based on this concept, immunogenicity of an antigen should depend on the number and diversity of carrier groups and one should be able to manipulate immunogenicity by adding or deleting carrier groups. This, in fact, is the case. Antibody response is suppressed by blocking carrier groups and it is enhanced by adding carrier groups. As examples of enhancement, autoantibodies were produced against muscle antigens and erythrocyte antigens by coupling to them carrier groups. These may be regarded as experimental models of autoimmune disease because animals producing tissue-specific autoantibodies showed typical autoimmune syndromes.

I. GENERAL OBJECTIVES

This investigation on the role of the carrier in antibody formation was prompted by the discovery that denatured calf thymus DNA and type III pneumococcal capsular polysaccharide, which are not immunogenic in rabbits, are converted into effective immunogens by complexing them to methylated bovine serum albumin (MBSA) as a carrier. The use of MBSA as a carrier was subsequently extended by us and by other investigators to the production of antibodies against other heterologous non-immunogenic polymers, including homopolymers of amino acids. At this time the decision was made to study in depth the function of a carrier in antibody formation in order to be able to use carriers as effectively as possible in the production of antibodies against biologically important haptens, to extend the use of carriers to homologous tissue antigens for the production of autoantibodies and the development of experimental models of autoimmune diseases, and to gain a better understanding of the mechanism of antibody formation in terms of the steps involved in which carriers play a role. Progress made toward the achievement of these general objectives is summarized next.

II. SUMMARY OF PROGRESS

A. Production of Antibodies Against Polynucleotides

When complexed to methylated bovine serum albumin (MBSA) as a carrier, polynucleotides are immunogenic in rabbits. The specificity of the antibodies produced is dependent on the base composition and the configuration of the polynucleotide in the immunogen preparation. The possibility of being able to use antibodies, specific for double-strand polyribonucleotides, in an immunochemical analysis of oligonucleotide-polynucleotide hybrids prompted a study of double-strand polyribonucleotides as haptens. Hybrids, formed by mixing poly A and poly U in a proportion giving maximal hypochromicity, were complexed to MBSA and injected into rabbits. The resulting antisera contained complement-fixing antibodies specific for hybrids of poly A and poly U, the extent of complement-fixation being proportional to the extent of hypochromicity. Neither poly C-poly G hybrids nor native double-strand calf thymus DNA were reactive as antigens with anti-(poly A-poly U), a clear indication that the helical configuration of the sugar-phosphate backbone of double-strand polynucleotides differs depending on the base composition. Also, rabbit anti-(poly C-poly G) reacted with poly C-poly G but not with poly A-poly U. Thus, antibody can distinguish between A-U and C-G base pairs. Rabbit anti-(poly A-poly U) reacted with mixtures of poly U and oligo A provided the oligo A contained at least 5 residues, an indication that penta A can hybridize in the presence of anti-(poly A-poly U) with polynucleotides having complementary pentauridylate sequences. Accordingly, a synthetic random copolymer of A and U (poly AU) was tested with penta A; it was unreactive. However, a mixture of poly AU and poly A did fix complement. These results imply that poly AU did not contain linear sequences of U with 5 or more residues and that

the antigenic determinant consists of a sequence of A-U base pairs fewer than 5 units in length. Thus, poly A and poly U may be used to probe the structure of polyribonucleotides for unpaired sequences of A and U. A sequence of 4 pairs suffices but the absolute minimum required may be smaller. With this knowledge regarding the specificity of anti-(poly A-poly U) serum, some tentative conclusions may be made about the structure of a yeast s-RNA tested as an antigen. It does not contain double-stranded regions with sequences of A-U base pairs more than 3 units long since it failed to react with anti-(poly A-poly U) serum. It does contain, however, unpaired sequences of A (of the order of 4 residues) that enable it to hybridize with poly U. Additional residues of A, present originally in a paired state, become available for complementation on denaturation of the s-RNA by heating at 100°C. Mixtures of poly A and s-RNA (native and denatured) were unreactive as antigens, an indication that the s-RNA contained little of its U in an unpaired state or the sequences of unpaired U were too short to form effective antigenic determinants.

B. Production of Antibodies Against Homologous Tissue Antigens

Muscle antigens. In a first attempt to develop experimental models of autoimmune diseases, one of a series of rabbits, primed with BSA-DNP (dinitrophenylated bovine serum albumin) and subsequently boosted with a DNP-conjugate of normal rabbit heart antigens, produced antibodies specific for rabbit skeletal muscle and heart tissue antigens and in addition developed a frank myasthenia gravis syndrome. To verify this initial finding and ascertain its reproducibility, a group of 20 rabbits were similarly primed and boosted. A transient state of muscle weakness was observed in 2 of the 20 animals. Little, if any, circulating antibody specific for rabbit tissue antigens could be detected. Apparently, the results of the first experiment could not be reproduced. In a final attempt, about a year after the start of this experiment, these rabbits were given a series of three intravenous injections of a DNP-conjugate of rabbit tissue antigen. Again there was no evidence of auto-antibody production or autoimmune disease. The DNP had apparently failed to function as a carrier, possibly because the priming with DNP had been too extensive and resulted in a large population of memory cells committed to the production of DNP-specific antibodies and effectively competing with cells able to make antibodies specific for rabbit heart antigens in the DNP conjugate. In fact, the sera of these rabbits, after being boosted with DNP-rabbit tissue antigens, contained large amounts of DNP-specific antibody. Although these animals had been primed originally with BSA-DNP, no BSA-specific antibodies were found. This finding implies that the secondary response primarily due to memory cells, is determinant-specific rather than antigen-specific.

Tumor tissue transplantation antigens. A primary objective was to determine the feasibility of using carriers to potentiate the immunogenicity of tumor-specific transplantation antigens in syngeneic hosts. As a model system, Rauscher Ascites Leukemia Cells (RAL), induced in BALB/c mice by the Rauscher Leukemia Virus, were tested as allografts in CF₁ mice. A single intradermal injection of live RAL sufficed to induce a cell-mediated immunity

in CF₁ mice, but it killed 100% of BALB/c mice within 14 days. Vaccines prepared with live RAL, RAL disrupted mechanically by freezing and thawing, and RAL frozen and thawed after conjugation to bovine γ -globulin, were compared for efficacy in CF₁ mice. The carrier-conjugated cells were least effective, presumably because the carrier portion blocked the antigenic determinants of the tumor transplantation antigens. The importance of the amount and nature of the carrier used was therefore investigated.

In order to assess the role of a carrier in initiating an immune response, ByG-DNP (dinitrophenylated bovine γ globulin) was precipitated by rabbit anti-ByG and by the basic protein MBSA (methylated bovine serum albumin). Both react with and block ByG determinants of ByG-DNP, thus reducing the number of its carrier determinants. Rabbits were immunized with ByG-DNP, rabbit antibody-aggregated ByG-DNP, and MBSA-aggregated ByG-DNP, and the sera of these rabbits assayed for DNP and ByG-specific antibodies. Antibody suppressed immunogenicity; whereas MBSA enhanced it. Both substances, however, suppressed antibody synthesis to determinants of ByG, the very determinants blocked by antibody and MBSA. The chief difference between rabbit antibody and MBSA lies in their immunogenicity in rabbits. MBSA is immunogenic and rabbit antibody is not. Thus, MBSA can compensate for the carrier determinants of ByG it blocks, whereas rabbit antibody can not. It seems, therefore, that the immunogenicity of an antigen depends on the number and kinds of carrier determinants it has and that the specificity of the antibodies elicited reflects those determinants that are accessible and free to interact with specific antibody-forming stem cells.

C. Means of Increasing the Efficacy of a Carrier

Based on the experimental fact that the antibody response to a hapten depends on the immunogenicity of the carrier to which it is conjugated, the assumption was made that a carrier should be more efficient in animals that have been primed to it. To test this assumption, rabbits were primed with BSA and subsequently boosted with BSA-DNP, and the response to DNP compared with the DNP response in normal non-primed rabbits given BSA-DNP for the first time. The response of primed rabbits was superior in terms of the early appearance of DNP-specific antibody and the maximal amount of antibody formed. A variation of this type of experiment was to prime rabbits with BSA-DNP and boost them with DNP conjugated to different unrelated proteins and homologous antigens. The response to these antigens was better in the primed animals than in normal non-primed animals. The explanation for this finding is that groups such as DNP, generally regarded as haptens, can also serve as carriers and do so more efficiently in animals primed to them. These results have provided important clues regarding the function of a carrier and have established a practical means of increasing the efficiency of a carrier.

D. Modification of the Immunogenicity of an Antigen

The specific objective was to ascertain the effect on immunogenicity of an antigen by reducing the number of its determinants. Two antigens were used: B γ G-DNP and sheep erythrocytes.

B γ G-DNP was precipitated by rabbit anti-B γ G and by MBSA. Both react with or block the B γ G determinants, which are the carrier determinants. The DNP groups are not affected. The result of aggregating B γ G-DNP with antibody or MBSA is to reduce the number of carrier determinants. Rabbits were immunized with aggregated B γ G-DNP and with soluble B γ G-DNP, and the response of these rabbits to DNP compared. Rabbit anti-B γ G suppressed the formation of DNP-specific antibody whereas MBSA enhanced its formation. The chief difference between rabbit antibody and MBSA lies in their immunogenicity in rabbits; MBSA is immunogenic and rabbit antibody is not. A reasonable explanation for the results obtained is that the carrier groups of MBSA compensate for the loss of B γ G carrier groups; rabbit antibody, lacking carrier function, can not do this. The conclusion, based on this study, is that the antibody response to any determinant of an antigen depends on the number of other determinants also present that can function as carrier determinants.

Sheep erythrocytes were coated with specific rabbit antibody, the objective being to determine the effect of blocking indiscriminately the determinants of an antigen. Mice were immunized with antibody-coated erythrocytes and with uncoated erythrocytes. The antibody response to cell surface determinants was suppressed by antibody in proportion to the amount of antibody used to coat the erythrocytes. True, coating of the erythrocytes with specific antibody reduced the number of carrier determinants, but the rabbit antibody used should have compensated for this loss of carrier determinants with its own carrier determinants since it is immunogenic in mice. A more likely explanation for the suppression of agglutinin synthesis is that the antibody also reduced the number of determinants needed to induce antibody formation. In essence, induction of antibody-forming stem cells to undergo differentiation and development requires the active participation of individual antigenic determinants as inducers.

E. Function of the Carrier

Results obtained and summarized above permitted, early in the course of these studies, the formulation of some rational conclusions regarding the function of the carrier in antibody formation and the steps of initiation and induction in the immune response.

An antigen is a substance made up of chemical groupings (determinants) against which antibody can be made in a host possessing antibody-forming stem cells specific for these determinants. The induction of such antibody-forming stem cells to differentiate and develop into clones of mature antibody-synthesizing cells requires the active interaction of these stem cells with

specific individual antigenic determinants. However, this interaction between antibody-forming stem cell and determinant is not effective unless it is mediated by carrier determinants, also present on the antigen. This postulated mediation most likely involves the participation of a second type of cell, a mediator cell, whose function it is to scavenge and concentrate on its surface antigens having carrier determinants and to transport these antigens to lymphoid organs. It is now known that the antibody-producing stem cells are bone marrow derived (B cells) and that the mediator cells are thymus-dependent (T cells). Here they attract antibody-forming stem cells that can interact with those determinants of the antigen not bound to a mediator cell and thus in a free reactive state. As a consequence of this specific interaction between mediator cell and antibody-forming stem cell, conceivably a derepressor-like substance might be transferred from mediator cell to antibody forming stem cell, thereby triggering the latter to undergo differentiation. In terms of this concept, a carrier is any molecule or substance that has carrier determinants, a carrier determinant being one that can interact specifically with mediator cells, and a hapten determinant is one that can interact only with antibody-forming stem cells. Thus, the key function of a carrier is to mediate the induction of specific antibody-forming stem cells by hapten determinants. This can explain why the antibody response to a hapten depends on the immunogenicity of the carrier to which it is conjugated, why the immunogenicity of an antigen depends on the number and diversity of its carrier determinants.

Since a carrier only mediates the induction of antibody-forming cells, it follows that induction requires specific stimulation by antigenic determinants and that the specificity of the antibodies formed will reflect only those determinants that are in a free reactive state capable of interacting with precursor antibody-forming stem cells.

F. Characterization of Carrier Groups

Basic proteins as carriers. Methylated bovine serum albumin (MBSA), a basic protein, has been used successfully to convert several classes of acidic non-immunogenic biopolymers (polynucleotides, homopolymers of amino acids, certain polysaccharides and homologous tissue antigens) into effective immunogens. The function of MBSA was assumed to be that of a carrier capable of reacting with carrier-specific cells needed to mediate the induction of antibody synthesis in hapten-specific cells. Subsequently, it was shown that MBSA can also modify the immune response to antigens that are themselves immunogenic and therefore do not require an external carrier. The observed effect of MBSA was not due to its ability to aggregate these antigens (BSA-DNP and BGG-DNP). Calf thymus histone and salmon sperm protamine, both of them basic substances but neither being immunogenic in rabbits, had essentially the same effect as MBSA, an indication that the carrier effect of MBSA in rabbits can not be entirely specific in terms of its immunogenicity, that is the specific interaction between antigenic groups of MBSA and carrier-specific mediator cells. This was further supported by the

finding that the effect of MBSA can not be distinguished from that of methylated rabbit serum albumin and methylated albumin of several other species. Since all these carriers are basic substances, it seems that non-specific charge interactions can also contribute substantially to the carrier effect. Ryser (Science, 159, 390, 1968) has shown that basic proteins associate readily with cell membranes and cause an increase in the rate of pinocytotic vesicle formation and of uptake in general.

DNP as a carrier. The dinitrophenyl group is a small molecule and constitutes a single antigenic determinant. It is immunogenic provided it is injected as a conjugate with a carrier, usually an immunogenic heterologous protein, and for this reason it - and other similar molecules - were designated as haptens by Landsteiner who pioneered the concept of carriers and haptens. The time has come, however, to redefine carrier and hapten in terms of their immunological function. A hapten is the inducer of antibody formation and functions by reacting with specific receptor sites on the surface of antibody-forming precursor cells. This induction apparently requires the cooperation of mediator cells that react, specifically or otherwise, with carrier groups associated with the hapten. Thus, a carrier molecule is one that contains chemical groups capable of reacting with mediator cells and a hapten molecule is one that contains chemical groups (antigenic determinants) capable of stimulating antibody-forming precursor cells to differentiate and proliferate into clones of antibody-forming cells. It follows from these definitions that an immunogen is a molecule that contains both carrier and hapten groups and that a group can serve both as carrier and hapten if it can react with both mediator cells and antibody-forming stem cells. In this context, a group such as DNP should serve as hapten and carrier if it is conjugated multiply to an immunologically inert molecule that contains neither carrier nor hapten groups. This prediction has been confirmed. The conjugation of DNP to rabbit serum albumin, which is inert in the rabbit, results in a molecule that is immunogenic in rabbits and the antibodies formed are specific for DNP. It also follows from the above definition of an immunogen that the greater is the number of DNP groups per molecule of rabbit serum albumin the larger should be the amount of antibody formed. This prediction has also been verified. On the basis of these results, it should be possible to use DNP as a carrier for polyvalent haptens, much as the basic proteins have been used. This has proven to be the case.

Function of the carrier in the secondary response. Results of previous studies had indicated that an antigen, when used as a carrier for a hapten in rabbits previously immunized to that antigen, stimulated "memory" cells (postulated cells believed responsible for a secondary response) to produce antibodies against the hapten as well as the carrier protein. These studies were extended to include the response of "memory" cells following their stimulation in vitro. Rabbits were primed to BSA (bovine serum albumin), and at different times thereafter they were sacrificed, their spleens removed, and suspensions of cells were prepared from them. Aliquots of these cell suspensions were incubated in vitro with BSA, derivatives of BSA, or unrelated protein antigens. The incubated cells,

washed free of the antigen(s) added, were transferred into x-irradiated HA/ICR mice. Their sera were tested individually for hemagglutinating antibodies by the method of passive hemagglutination. The spleen cells of primed rabbits could be stimulated to produce antibodies only by BSA, the priming antigen, and by conjugates of BSA. More importantly, stimulation resulted in the production of antibodies not only against determinants of the priming antigen, as expected, but also against different unrelated determinants provided these were conjugated to the priming antigen. These results are consistent with those observed in intact rabbits, and the general conclusions are: (1) an antigen can function as a carrier for new determinants in an animal previously immunized with it, (2) the stimulation of "memory" cells is specific, and (3) "memory" cells need not be committed to determinants of the priming antigen and can therefore participate in the synthesis of antibody to new determinants that are conjugated to the priming antigen.

G. Induction of Antibody Synthesis

The initiation of antibody formation depends on the interaction between immuno-competent cells and antigen. However, the induction of antibody synthesis and the specificity of antibodies formed seem to depend on the number and kind of determinants carried by the antigen, in which case one should be able to suppress the induction of antibody to a given determinant of an antigen by blocking preferentially that determinant. This we have been able to do. BGG-DNP was selected as the antigen because it carries two classes of determinants, those in common with native BGG and also some denatured determinants involving the hapten DNP. We could therefore use antibodies or antibody fragments to BGG to block preferentially the native determinants of BGG-DNP. Rabbits were immunized with complexes of BGG-DNP antibody, BGG-DNP-antibody fragments, or BGG-DNP, and the sera were tested by quantitative complement-fixation and precipitation for antibodies specific for BGG and BGG-DNP. The results clearly showed that antibodies to BGG suppressed preferentially the formation of antibodies to native determinants of BGG-DNP. These data support the hypothesis that induction of antibody synthesis to a given determinant depends on the accessibility of that determinant to an antibody-forming cell. The molecule or complex of which a determinant is a part serves only as a carrier for that determinant, enabling it to interact with appropriate immunocompetent cells that can process it.

H. Mechanism and Regulation of the Immune Response

The current consensus is that antibody formation in response to an antigen results from the interaction of at least 2 different cell types, a thymic-derived mediator cell (T cell) and a bone marrow antibody-producing cell (B cell). The mediator cell reacts with the carrier portion of the antigen, and in so doing participates in the induction of antibody synthesis by the hapten groups of the antigen. Some fundamental questions to be answered are: (1) What constitutes a carrier group of an antigen and what is the nature of the reactive

sites on the surface of the T cell? (2) Can a hapten group function also as a carrier group? (3) What is the relationship between immunogenicity of an antigen and the number and kinds of groups it contains? (4) What are the properties and functions of the T cell? (5) Is acquired immunological unresponsiveness the result of a deficiency of T cells? and (6) Can the immune response be modified and regulated through the use of carriers and selective reagents?

The results of research already summarized provide answers to the first three questions. Next to be summarized is the research concerned with the remaining questions.

Characterization and isolation of carrier-reactive T cells. Major classes of immunocompetent cells are the thymus-dependent cells (T cells) and the bone marrow-derived cells (B cells). The latter are the antibody-producing cells, are fixed in lymphoid organs, and they do not in general respond on direct reaction with antigen. Their activation is dependent instead on the cooperative action of T cells that circulate, scavenge and concentrate antigen on their surface, and transport the antigen efficiently to lymphoid organs where interaction with B cells can occur. A key function of T cells would seem to be that of a carrier or vehicle. For this reason, they are denoted as carrier cells and the chemical groups of the antigen responsible for its binding to T cells are called carrier groups. Being carrier cells, T cells are intermediate cells and are expected to function very early in the course of the immune response. Thus T cells with bound antigen should accumulate early in lymphoid organs, such as the spleen, and they should be functional in activating B cells. These expectations have been verified experimentally and it has been possible to isolate and characterize them.

Spleen cells, taken from C57Bl/6J mice as early as 4 hours after they had been injected with sRBC (sheep red blood cells), contained antigen-reacted T cells capable of passively inducing an immune response in syngeneic recipients, measured in terms of circulating hemagglutinating antibody and plaque-forming spleen cells (PFC). The function of these cells was shown to depend on cell viability, association with antigen, and interaction with histocompatible radiation-sensitive recipient lymphocytes. This study has been continued, attaining additional key information.

Functional antigen-reacted T cells peak at 4 hours following the i.p. injection of sRBC, based on analysis of spleen cells as a function of time for carrier function (induction of antibody formation on transfer to syngeneic recipients) and for Θ and sRBC antigen markers on their surface (susceptibility to the complement-dependent cytotoxic action of rabbit anti- Θ and rabbit anti-sRBC). For this reason, 4 hr. SS (stimulated spleen cells taken 4 hours after injection of sRBC) were used in this study. The addition of 4 hr. SS cells to nylon fiber conjugated with sRBC-specific rabbit antibody resulted in the selective binding of functional cells. At least 50% of the bound cells were Θ -positive, whereas only about 20% of cells in the whole spleen were Θ -positive, an indication that cells having bound antigen were largely T cells.

When the bound cells, either attached to the antibody-conjugated fiber or plucked from it, were cultured in vitro with normal syngeneic spleen cells, PFC developed by day 4. The yield of PFC compared favorably with that obtained in cultures of unfractionated 4 hr. SS. No PFC developed if: (1) normal spleen cells were omitted from the culture of fiber-bound cells and (2) if both the normal and 4 hr. SS cells were given 1000 R of x-radiation. The function of 4 hr. SS cells was therefore not affected by x-radiation. However, exposure of these cells to rabbit anti-sRBC rendered them impotent. In contrast, exposure to antibody had no effect on spleen cells taken from mice given sRBC 24 or 48 hours earlier at a time when induction of B cells had already occurred and was no longer dependent on interaction of B cells with sRBC-associated T cells.

Based on the results to date, it may be reasonably concluded that among the functional cells found in the 4 hr. SS preparation, a major portion are T cells (Θ -bearing) having sRBC determinants (reactive with rabbit anti-sRBC) on their surface that can bind to specific receptors on B cells, the interaction of such antigen-associated T cells with B cells results in activation and proliferation of B cells to antibody-forming PFC. The antigen carried by the T-cells is probably processed antigen, since rosettes (lymphocytes surrounded by RBC) are not seen in suspensions of 4 hr. SS. Since antigen-associated T cells are found in the spleen by 4 hours after injection of sRBC and their number does not increase during the next 24 hours, it seems that these T cells do not derive from antigen-activated cells but are instead pre-existing cells that can bind antigen to their surface and circulate to lymphoid organs where they can activate B cells with receptors for the antigen determinants that these T cells carry. Because the fraction of functional spleen cells that bear Θ -antigen and sRBC determinants peaks at 4 hours after injection of sRBC, it seems that T cells carrying antigen accumulate rapidly and early in the spleen as they circulate through various lymphoid organs. The fact that these antigen-carrying T cells function following their exposure to 1000 R of x-radiation suggests that DNA synthesis and cell division of these cells is not needed for induction of PFC.

Cellular basis for acquired immunological unresponsiveness. Rabbits and mice were rendered unresponsive to BSA-DNP by x-irradiation or by a regimen of sub-immunogenic doses of antigen. During the period that the animals were unresponsive to DNP conjugated to BSA, the original protein carrier, these animals were shown to be responsive to DNP provided it was conjugated to a new carrier protein to which they were responsive. The fact that tolerance to DNP could be abrogated by a switch to a new carrier clearly indicates that acquired tolerance, induced as above, involves a deficiency of carrier-reactive T cells. DNP-reactive antibody-forming B cells are also involved, however, because the DNP-reactive antibodies formed by tolerant animals had an average affinity significantly lower than antibodies produced by normal untreated animals.

Rabbits, unresponsive to BSA-DNP in terms of humoral antibody, also failed to develop delayed skin reaction to BSA-DNP or to RSA-DNP following immunization with BSA-DNP, an indication that they were also unresponsive

with respect to cellular immunity. A group of unresponsive animals that had been immunized with DNP conjugated to a new carrier protein did develop delayed skin reactions to RSA-DNP. This suggests that activation of cellular immunity may also depend on the cooperative action of carrier-reactive cells, as does the activation of humoral antibody.

Regulation of the immune response and tumor immunity. The immune response is dual in nature, and it can be either cellular or humoral. Manifestations, such as delayed hypersensitivity, graft vs. host and homograft rejection, are primarily due to cellular immunity. The basis for tumor immunity is also predominantly cellular, and because of the increasing evidence that humoral antibody can block cell-mediated tumor immunity it has become critically important to be able to selectively stimulate a cellular immune response to tumor-specific antigens in order to achieve effective tumor immunity through vaccination. This prompted us to attempt to regulate the immune response to tumor tissue antigens through manipulation of the antigenic material in terms of the amount and form administered, its route of administration, its viability and its modification by chemical treatment or by passaging through allogeneic and xenogeneic hosts.

Since the immune response to syngeneic tumors is generally weak and difficult to assess, the response of competent hosts to tumor tissue as an allograft was first studied instead. The system used was Rauscher virus-induced Balb/c ascites cells in allogeneic Ha/ICR mice. Cellular immunity is easily measured in terms of the rate of rejection of viable tumor allografts, and humoral antibody can be assayed as Balb/c erythrocyte-binding immunoglobulins since Balb/c ascites cells and erythrocytes share strain-specific histocompatibility antigens. The intradermal and subcutaneous routes proved best in inducing cellular immunity. For all routes of administration tested, increasing the antigenic mass resulted in increased amounts and rate of synthesis of circulating antibody. Isoantiserum, from Ha/ICR mice immune to BALB/c ascites tumor, was passively transferred to Ha/ICR mice as a means of blocking initiation of the immune response to Balb/c ascites cells. The cellular response could be blocked, but not completely, if the antiserum were transferred within 7 hours after injecting the immunogen. In contrast, the humoral antibody response was significantly blocked, and it was significantly suppressed even when the transfer of antiserum was delayed until 12 to 24 hours after the immunogen. Thus, initiation of the cellular response to the point where it no longer is antigen-dependent is more rapid than is the initiation of the humoral response. Despite the rapidity of initiation of the cellular response, it takes about 6 days for its development in terms of the ability of the immunized host to reject a challenge viable tumor allograft. Both initiation and development of cellular immunity to a tumor allograft is more rapid compared with humoral immunity. Another finding was that x-irradiated non-dividing tumor cells were markedly less immunogenic than viable cells; 100 to 1000 more x-irradiated cells, given as a series of 3 weekly injections, were needed compared to a single inoculum of viable cells. The difference between the two preparations might be due to the increase in antigenic mass resulting from division of viable cells, but, assuming a reasonable cell generation time and

knowing the time required for development of immunity and rejection of the viable cells used to immunize, the increase in antigenic mass could be calculated and was not sufficient to account for the immunogenicity of the viable cells. Possibly, x-irradiation causes the tumor alloantigens to undergo alteration, or these antigens are exposed only in certain stages of the cell cycle.

With the knowledge that inactivation of a tumor allograft results in a decrease in its immunogenicity and that tumor immunity can be induced in syngeneic hosts by a viable tumor graft provided the tumor is excised after an appropriate time interval, means were explored to attenuate, rather than to inactivate, syngeneic tumors (Balb/c virus-induced ascites cells and C57 Bl/6J methylcholanthrene-induced sarcomas) and test them for their viability and their ability to induce tumor immunity without necessitating excision. Minimal tumor mass and the intradermal or subcutaneous routes were used, based on the experience with tumor allografts. Methods used to attenuate the test tumors were: (1) conjugation to heterologous proteins, and (2) passaging in allogeneic and xenogeneic hosts. The tumors so modified proved either too viable or biologically inactive but not immunogenic. Alternative means for attenuating syngeneic tumors need to be explored.

The possibility that rejection of syngeneic tumor grafts might be mediated in part by non-specific cellular responses prompted a study of the response of C57Bl/6J mice to syngeneic tumors mixed with adjuvant materials or with unrelated antigens (allogeneic tumor cells or normal spleen cells, picryl chloride which was used to sensitize and provoke a delayed skin reaction in the site of injection of the syngeneic tumor). Little effect, if any, was noted on the syngeneic tumor progression and the median survival time of the mice. This suggests either that activation of a non-specific cellular response by adjuvants and unrelated antigens was minimal or that it was inadequate in causing rejection of a syngeneic tumor.

Viral etiology of acquired hemolytic anemia. The NZB strain of mice spontaneously and predictably develops autoimmune disease. One manifestation is the conversion, at about 3 months of age, of their erythrocytes to Coombs positive, due to the production of erythrocyte-reactive autoantibodies. This autoimmune response is associated with a response to indigenous Gross leukemia virus. One possibility is that the virus alters the antigenicity of the erythrocytes and/or functions as an immunological carrier. This prompted a study of the immunogenicity of influenza virus-coated syngeneic erythrocytes in C57Bl/6J mice. A series of 4 or 5 weekly i/p injections of such erythrocytes resulted in an autoimmune response, assayed in terms of Coombs positive erythrocytes, erythrocyte-binding immunoglobulins in the plasma, and plaque-forming spleen cells. The autoantibodies were both species- and strain-specific, in contrast to the antibodies in NZB which are species-specific only. The immunogenicity of virus-coated syngeneic erythrocytes was optimal at one-half the minimal amount of virus needed to agglutinate the erythrocytes. Virus, inactivated by heating or by periodate oxidation, was effective but less so than viable virus in converting syngeneic erythrocytes to

immunogens. Inoculation of mice with free viable virus was not effective, however, indicating that the virus must be physically associated with the erythrocytes. The mice producing autoantibodies showed evidence of hemolytic anemia. These results admit the possibility, but do not prove, that natural acquired hemolytic anemia might have a viral etiology.

III. TRAINEES SUPPORTED

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IV. PUBLICATIONS

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