CROSS-STIMULATION OF MONOCLONAL ANTIBODIES IN ANAMNESTIC RESPONSES

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The identification of cross-reactions between related but distinct antigens during anamnestic responses led to the concept of "original antigenic sin" (1-3). Such cross-reactivity noted first for antibodies to different influenza virus strains was also observed for antibodies to the haptens dinitrophenyl (DNP) and trinitrophenyl (TNP) (4), and to sulfanilate-metanilate haptenic groups (5).

In the system employing two strains of influenza virus as cross-reacting antigens, the phenomenon was interpreted as a restricted anamnestic response to the primary antigen mediated by a trapping mechanism deflecting antigen from one kind of cell to another (3). Chronologically analyzed, this process involved an early production of cross-reactive secondary response antibody followed by the primary response to the boosting antigen (3).

Antibodies stimulated by either DNP or TNP protein conjugates had a high degree of immunological specificity. The anamnestic recall phenomenon in this system was explained as a process of degeneracy of the immune response reflected by high affinity antibodies with low specificities (4).

The serological group specificity of streptococci results from distinct polysaccharide moieties characteristic for every group of streptococci (6, 7). For example, the group specific polysaccharides of A (A-CHO) and A-variant (Av-CHO) streptococci share a branched backbone of rhamnose. They differ, however, by the terminal N-acetylglucosaminide residues in Group A streptococci linked \( \beta 1 \rightarrow 3 \) glycosidically to the rhamnose side chains. This hexosamine moiety confers serological specificity to the Group A carbohydrate (6, 7). The A-variant polysaccharide, composed predominantly of rhamnose, has its cross-reactive serological representation in di- and trisaccharides of rhamnose of unknown linkage (6, 7).

Since antibodies of restricted electrophoretic mobility are inducible against both the streptococcal Groups A and A-variant polysaccharide (8, 9), the

Abbreviations used in this paper: A-CHO, streptococcal Group A polysaccharides; A-CHO tyr \( 125^I \) and A-CHO tyr \( 131^I \), streptococcal Group A polysaccharides tyraminated and either \( 125^I \) or \( 131^I \) labeled; Av-CHO, streptococcal Group A-variant polysaccharide; Av-CHO tyr \( 125^I \) and Av-CHO tyr \( 131^I \), streptococcal Group A-variant polysaccharide tyraminated and either \( 125^I \) or \( 131^I \) labeled; DNP, dinitrophenyl; DNP- and TNP-BGG, dinitro- and trinitrophenylated, respectively, bovine gamma globulin; IEF, isoelectric focusing; 1° immunization, primary immunization; 2° immunization, secondary immunization; TNP, trinitrophenyl.
system lends itself for reinvestigation of the phenomenon original antigenic sin. The antibodies induced by either antigen were compared on the basis of binding specificity, molecular restriction, and isoelectric focusing (IEF) characteristics documented by comparative IEF in analytical polyacrylamide gels.

**Materials and Methods**

*Animals and Immunization.*—BALB/cJ mice were used throughout this work since they were known to produce high levels of restricted antibodies to the streptococcal Group A antigen (9-11). They received a primary course of immunizations with Group A streptococcal vaccine (Group A streptococci, strain J17A4), hence referred to as course A, and 5-9 mo later a primary course of injections with two strains of Group A-variant streptococcal vaccine (Group A variant streptococci, strain A486 var. M- and strain K43 var.); all strains were kindly supplied by Dr. R. C. Lancefield, The Rockefeller University, New York. This course will be referred to as course Av and course Av' respectively. Each immunization course was given intraperitoneally for a 4-wk period (10, 11). Another group of mice was, after a primary course A vaccination, challenged secondarily with Type III pneumococcal vaccines (12). Antisera were obtained 5 days after the last injection and again several days later.

Two control groups of mice received either a primary (course A1°) and secondary (course A2°) immunization with Group A vaccine or a similar immunization with Group A-variant vaccine (course Av1° and 2°). Antisera were obtained as above.

*Quantitation of the Immune Response.*—Antibody levels were estimated by the amount of Ig absorbed with homologous streptococcal cells measured by a combination of microzone electrophoresis and protein determination (10). A modified Farr assay (13) was used for direct quantitation employing tyraminated $^{125}$I labeled Groups A (A-CHO tyr $^{125}$I) and A-variant (Av-CHO tyr $^{125}$I) polysaccharides (11) obtained by the hot formamide extraction of streptococcal cell walls (14).

*Isoelectric Focusing.*—IEF was carried out by the method of Awdeh et al. (15). This technique is a fast and convenient assay to determine the quality of specific antibodies in immune sera with regard to their degree of heterogeneity if combined with an antigen-binding assay. In this case, $^{131}$I-labeled tyraminated polysaccharide antigen (A-CHO tyr $^{131}$I and Av-CHO tyr $^{131}$I) was used to develop the patterns (11). The technique also allows inhibition of the specific Group A reaction by preincubation with 5% N-acetyl-glucosamine solution which will compete for the antibody-combining sites with the radioactively labeled group carbohydrate added subsequently.

In the sets of experiments shown here, identical amounts (e.g., 10 μl) of course A and course Av or Av' antisera from individual mice were focused in juxtapositions and reacted either with A-CHO tyr $^{131}$I or Av-CHO tyr $^{131}$I (10 μCi per plate, time of exposure 23 h). Although qualitative, this procedure permitted direct comparison of the relative amounts of cross-stimulated and/or cross-reactive monoclonal and multyclonal antibody populations.

**RESULTS**

Immunization of BALB/cJ mice with either Groups A or Av streptococcal vaccine for the induction of 1° and 2° immune responses associated with high levels of restricted antipolysaccharide antibodies leads basically to a similar observation as described for the rabbit (8, 9). When Av' vaccines were used as antigens the response, although highly restricted for the variant antigen, was usually lower in quantitative terms than after Av-vaccine immunizations. Secondary immunization is generally characterized by a pronounced expression
of molecularly restricted antibodies already identified after 1° immunization. Two representative examples were depicted (Fig. 1). The identical IEF patterns of monoclonal antibodies raised in an individual mouse, e.g., clone products in course Av1° and 2° antisera with IEF points (pI) of pH 6.7–7.2, must be regarded as evidence for the expression of identical antibody molecules at different times of immunization. Preboost antisera are generally devoid of significant levels of these monoclonal antibodies. If, in rare cases, they are demonstrable, they are present in levels at least 10X lower. Support for the interpretation that identical clones are triggered during both immunizations also comes from

the rabbit model, where an individual rabbit's monoclonal antibody following both 1° and 2° immunization was indistinguishable by IEF and also carried the same idiotypic specificity (16).

The notion of this control experiment is fundamental for the interpretation of IEF patterns developed with mouse antibodies raised by immunization course A followed by course Av or course Av'. Such data are depicted in Figs. 2–6.

Mouse sera I–IX (Figs. 2–6) are representative for 40 mice doubly immunized first with Group A vaccines and then with Groups Av (sera I–VII) or Av' (sera VIII and IX) vaccines. All nine mice shown here responded with restricted antibodies to the A-CHO (patterns I–A to IX–A) (Figs. 2, 6), and subsequently 21 out of 30 course Av mice (Fig. 4: 5 out of 7 mice) and 5 out of 10 course...
Fig. 2. Analytical IEF patterns of 10 μl of mouse antisera I–VII. These mice had received a 1st immunization course with group A streptococcal vaccines (A) and a 2nd course with group Av streptococcal vaccines (Av). The patterns were developed with A-CHO tyr 131I and exposed to the X-ray film for 23 h.

Fig. 3. Analytical IEF patterns of 10 μl of mouse antisera I–VII as in Fig. 2. Before the addition of A-CHO tyr 131I, the gels were exposed to 5% N-acetyl-glucosamine. Exposure time to the X-ray film was 23 h.

Fig. 4. Analytical IEF patterns of 10 μl of mouse antisera I–VII as in Fig. 2. The patterns were developed with Av-CHO tyr 131I and exposed to the X-ray film for 23 h.

Fig. 5. Analytical IEF patterns of 10 μl of mouse antisera I–VII as in Fig. 2. Before the addition of Av-CHO 131I, the gels were exposed to 5% N-acetyl-glucosamine. Exposure time to the X-ray film was 23 h.
Av' mice expressed defined monoclonal antibodies to the variant antigen. Individual mouse antisera differed by both the degree of restriction and the number of stimulated monoclonal antibodies dominating the diffuse background of polysaccharide-specific antibodies.

Comparison of the course A and course Av as well as Av' antisera obtained from individual mice (Fig. 2, 6) revealed monoclonal A-CHO-specific antibodies (represented by a three- to four-band pattern mostly) which were continuous into the course Av and course Av' antisera (I, II, III, VI–IX). The intensity of banding with labeled A-CHO was usually less strong in the course Av and Av' antisera. Since identical amounts of antisera were applied, this was taken as an indication that generally clones were stimulated less intensely by the heterologous antigen. The very marked A-CHO specific clone of antiserum III-A (pI 6.4–6.7), however, is equally prominent in the course Av antiserum. In antiserum II-Av two clones (pI 7.5–8.2, Fig. 2) and antiserum IX-Av' two to three A-CHO-specific clones (pI 6.5–7.4, Fig. 6) appeared to be stimulated to an even higher production than in the homologous antisera II-A, and IX-A, respectively.

In general, boosting with the closely related but distinct Av or Av' streptococcal vaccine triggered mainly those A-CHO-specific clones which had risen

Fig. 6. Analytical IEF patterns of 15 µl of mouse antisera VIII and IX. These mice had received a 1° immunization with Group A streptococcal vaccine (A) and a 2° course with group Av' streptococcal vaccine (Av'). A preboosting bleed (p) was included for comparison which contained practically no detectable A-CHO antibody. The patterns were developed with A-CHO tyr 111I and exposed to the X-ray film for 10 h.
to high levels already during $1^\circ$ immunization with the homologous antigen. A somewhat different situation was observed in antiserum V-Av whose companion course A antiserum was rather complex in terms of A-CHO-binding specificities. Antiserum IV-Av represents an example of those five out of 30 mice where no stimulation of group A-CHO-specific clones occurred upon immunization with Av-variant vaccines.

Incubation of a companion IEF gel with 5% N-acetyl-glucosamine (Fig. 3) (this hexosamine is inhibitory for the Group A-specific precipitin reaction [6, 7]) for 20 min at 37°C before the addition of the labeled A-CHO rendered all but one distinct clone (antiserum VII-Av, pI 6.8–7.1), covered in an intense smear of the A-CHO specific antiserum, specifically inhibitable. Under conditions depicted here, antisera IV-A and III-Av best demonstrated the degree of specificity (compare clones at pI 7.7–8.4, 6.4–6.7 and 7.4–8.5, respectively). However, if only 1 μl of antiserum I–VII were subjected to the same conditions, all strongly expressed clones in the experiment of Fig. 3 diminished substantially, indicating an excess antibody in 10 μl of antiserum over the inhibiting hapten concentration. Thus, free antibody was available to bind labeled A-CHO.

Testing for the occurrence of restricted variant-specific antibodies in both course Av and course Av' antisera was performed in a parallel set of experiments. Thus, if 10 μl of each course A and course Av, respectively, Av' antiserum were developed after IEF with Av-CHO tyr $^{131}$I, which was shown to consist almost exclusively of rhamnose (6, 7), patterns were obtained as depicted for Av-antisera in Fig. 4. Clear-cut monoclonal antibody populations were stimulated which were absent in the course A antisera, however. This observation also held for the Av' antisera where only after the course Av' homogeneous antibodies binding specifically to the radiolabeled Av-antigen were identified which were absent in the course A antisera of these mice.

The degree of specificity for the Av-CHO is exclusive for all strongly expressed antibody clones (antisera II-Av and VI-Av), as well as Av' antibody clones not shown here. Some slightly cross-reactive and very minor clones were only present in the weak antisera I-Av and I-A as well as VII-Av and VII-A developed clearly only after doubling or tripling of the exposure time. As a result of this variant polysaccharide specificity, 5% N-acetyl-glucosamine added before the addition of Av-CHO tyr $^{131}$I was ineffective as inhibitor (Fig. 5).

A modified Farr assay (13) was designed to quantitate specific antibodies in course Av and Av' immune sera by binding to the isolated $^{131}$I-labeled A-CHO and Av-CHO. The experiments generally revealed an excess of A-CHO specific antibodies over the Av-CHO-binding antibodies, corresponding to the qualitative data obtained by IEF. However, this was not the case for antiserum IV-Av and V-Av.

The strict serological specificity for the homologous group polysaccharide antigens was furthermore documented by absorption studies with the course
Av antisera which employed excess of Group A and Group Av streptococcal cells used as vaccines for immunization. Absorption with Group A streptococcal cells removed all but the Av-specific antibodies, and conversely absorption with Group Av streptococcal cells removed selectively the Group Av-specific antibodies.

In order to eliminate the possibility that the Group A-CHO-binding antibodies in the course Av-antisera were binding to the N-acetyl-glucosamine moiety of the mucoprotein (antibodies directed to the sugar moieties of the mucoprotein were reported [17]) and that renewed stimulation of defined monoclonal A-CHO specific antibodies was due to such cross-reaction, 50 µl of antisera VIII-Av' and IX-Av' were absorbed with 3 mg each of O90R mucoprotein for 60 min at room temperature and 12 h at 4°C. The antiserum was recovered by centrifugation and again focused on two pH 5-10 gels, one of which was specifically stained with the radiolabeled A-CHO and the second with radiolabeled Av-CHO. Since no absorption of the A-CHO-specific clones which were triggered during course A and again during course Av' immunization was achieved by the sugar moiety of the mucoprotein preparation, it was concluded that this part of the streptococcal cell wall did not cross-react to a significant degree with these particular monoclonal A-CHO antibodies. From this result we would conclude furthermore that their restimulation during the course Av' immunization was mediated by some other mechanism rather than cross-reaction via a possible N-acetyl-glucosamine determinant shared by gram-positive and gram-negative bacteria (17).

To further support this interpretation, 10 mice received after a course A immunization 3–7 mo later an intraperitoneal injection course with Type III pneumococcal vaccine. If the N-acetyl-glucosaminide residue of the sugar moiety in the mucoprotein of these bacteria was cross-stimulating A-CHO-specific antibody clones revealed during the course A immunization, these were expected to be restimulated under these conditions. This was not the case.

DISCUSSION

In the production of standard antisera to streptococcal Groups A and A-variant, cross-reactions between these related but serologically distinct antigens may be encountered. Two explanations for such an event are at hand: (a) two antigens share common determinants which are recognized by the immunized animal, and (b) cross-reactivity is a function of cross-stimulation of two antibody populations in the antiserum, one specific for one and the other for the related antigen. This is in essence the phenomenon of the anamnestic response as it was observed for various strains of influenza viruses (1–3), the DNP and TNP ligands (4), sulfanilate-metanilate haptenic groups (5), and some serum proteins (8–20).

2 This preparation was derived from the serological Group B streptococcal strain O90R.
For the streptococcal Group polysaccharides A and A variant, the case can be carried further. Removal of the terminal N-acetyl-glucosaminide residues from the A-CHO by an N-acetyl-glucosaminidase changes the serological and chemical properties of the polysaccharide into those of a typical Av-CHO (6, 7). Thus, the situation for probing the phenomenon of original antigenic sin appears favorable if it could be shown that antibody populations triggered are of strict specificities for either antigen and not the result of a shared immunodominant determinant.

This experimental system has an additional advantage in that homogeneous antibodies can be raised to both the A-CHO and the Av-CHO in rabbits (8, 9) and in mice (10, 11, 21, 22). Consequently, the phenomenon of the anamnestic response could be investigated on the level of monoclonal antibodies with clearly defined IEF spectra.

From the data presented here, it is apparent that the overwhelming quantity of antibody raised against either streptococcal antigen is of exquisite specificity. It appeared that the major fraction of the A-CHO-specific antibodies of restricted molecular properties was inhibited to bind the A-CHO tyr 131I by the addition of excess N-acetyl-glucosamine and thus directed to this determinant, which is characteristic for Group A-specific antibodies (6, 7). Cross-reacting antibodies with the Av-CHO were, if present, close or below detection level even with the rather sensitive radioimmuno assay. This was taken as evidence for nonoverlapping immunodominant determinants, despite the fact that both polysaccharides share a backbone of rhamnose, presumably indistinguishable if the proper determinants are made available to react with the antibody or to stimulate its production by contact with the antigen-sensitive cell.

With respect to this exclusive serological specificity, a similar conclusion was reached for DNP-BGG and TNP-BGG employed as cross-reacting ligands for cross-boosting. In those experiments also, a virtually nonoverlapping set of antigen-sensitive cells was stimulated (4).

The extraordinary degree of specificity and the lack of cross-reaction was very clear for the monoclonal antibodies in the antisera obtained after immunization with A-variant and A-variant' vaccines as the boosting antigens. The lack of cross-reactivity between the haptenes m-aminobenzoic acid and m-sulfanilic acid at the level of the antibody-secreting cell was recently emphasized. Nevertheless, the recall phenomenon could be induced in C57BL mice by successive immunizations with these haptenes (23). In our experiments, precisely those plasma cell clones were restimulated to yield a dominant secondary-type response that had dominated the pattern in the antisera raised with the primary immunogen, although the radioimmunoassay rendered them as noncross-reactive when isolated Av-CHO was used. Thus, they met all requirements to fulfill the recall phenomenon (3) which postulates that a noncross-reacting antigen, lacking in this case precisely the immunodominant N-acetylated hexosamine, provokes a response to an antigen experienced previously by the individual. In addition, the immune responses to the Av-CHO also met the requirements of the phenomenon: they
were highly and only selective for this antigen which is so totally mimiced by the A-CHO once devoid of the terminal N-acetylated hexosamine moiety (7).

The possibility that the Av-CHO antigen may possess N-acetyl-glucosamine moieties immunogenically active and thus constituting A-CHO determinants, since revertants from the A486 var. strain to A-CHO-carrying bacteria were observed (24), seems unlikely for the following reason: immunization with strain K43 var. led to an identical observation made with course A486-variant antisera. Furthermore, growth of the bacteria was exclusively performed at 37°C at which temperature no revertants were observed (24). Appropriate absorption studies with the streptococcal Av-CHO cells used as vaccine and with mucopeptide which also contains N-acetyl-glucosamine, furthermore excluded this alternative.

The typical secondary-type response cannot rest in a qualitative change of the antibodies triggered: they are identical. It must rest on the state of memory and/or antibody-secreting cells developed by the first proliferative process to a particular level; restimulation at this level requires an alternative explanation to mere cross-reactivity with N-acetyl-glucosamine-containing determinants.

The argument was raised that original antigenic sin was the example of degeneracy in the immune response; a process by which specificity is sacrificed with low affinity antibodies to gain antibodies of high affinity associated with greater cross-reactivity which are also more heterogeneous (4). Clearly, in the system of anamnestic response to streptococcal polysaccharide antigens, this is not the case. The monoclonal populations of antibody raised by the anamnestic response are highly specific for the homologous antigens, and the IEF patterns of the immune sera are reduced in complexity rather than increased.

These data then furnish evidence for the view that the secondary response, resulting from injections with nonhomologous antigen (3) is specific and clonally selective for the primary homologous antigen and this occurs as effectively and sometimes even better than the response to the primary course of immunization. The underlying mechanism may simply be immunogenic reactivation of persisting antigen (25). This possibility, however, appears unlikely because a secondary immunization course with the serologically unrelated Type III pneumococcal vaccine was not stimulating for the A-CHO-specific monoclonal antibodies so extensively produced during the primary course A.

An alternative explanation appears to be more attractive: in the system presented here where extensive in vitro cross-reactions are lacking, the situation is reduced to clonal dominance and persistence at the level of some kind of memory cells which are then reactivated for the anamnestic response. Clearly this has to do with the number and possibly also the efficiency of either the antigen-sensitive and/or the antibody-secreting cells of a particular clone. Efficiency in our view would simply be an expression of the difference in threshold required for triggering of newly stimulated proliferation and antibody
synthesis and secretion as compared to the primary stimulus, and is thus probably a function of a particular property of the membrane expressing specific Ig receptors. Low affinities recognizing the common rhamnose backbone of both antigens not measurable with our conventional antigen-binding techniques might still reflect the relatedness of the antigens. This image of the original antigen in which the terminal $N$-acetylated glucosamine is not only the determinant but rather the immunodominant group, might suffice as the starting-off signal for synthesis and secretion of antibody specifically combining with the $\beta_1 \rightarrow 3\ N$-acetyl-glucosamine determinants. Only in this sense would we interpret the response as deflected (3); however, this does not apply to the quality of antibody still highly specific for the original antigen contact.

Recently Ivanyi ascribed the mandatory role in the recall phenomenon to the thymus-derived cell (26). Although both thymus- and bone marrow-derived cells are required for the induction of the Group A-CHO response in the immunologically virgin thymus cell reconstituted nude mouse (9-10), in our opinion the phenomenon of the anamnestic response depends on the second, truly efferent level of the immune response: here the bone marrow-derived cells as the antibody-secreting cell may be the only target for the turn-on phenomenon due to a special arrangement and/or flexibility of the antibodies functioning as receptors. In the streptococcal system the recall phenomenon is therefore to be formulated as a reflection of cross-stimulation rather than cross-reaction as a typical secondary response of persisting antibody cell clones.

**SUMMARY**

Immunization of BALB/c mice with Group A streptococcal vaccines leads to the induction of high levels of monoclonal antibody populations. Subsequent immunization of these mice with Group A-variant streptococcal vaccines induces a significant level of monoclonal antibody of A-variant antigen specificity revealed by labeled group A-variant polysaccharide. During these course $Av$ and $Av'$ immunizations, the monoclonal Group A-specific antibodies were also restimulated to levels usually higher than the variant-specific antibodies. With two exceptions, these homogeneous antibody populations were not cross-reactive in vitro with the related antigen. Such cross-stimulation of monoclonal antibodies was interpreted as a function of particular membrane properties of the Ig receptor-bearing memory cells which, for restimulation, would only require the structurally closely related antigen which serves as a backbone to the original antigen, and not necessarily the exact fit of the homologous immunodominant group.

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