

HAPTEN-SPECIFIC HEMOLYTIC PLAQUE ASSAYS USUALLY FAIL TO DETECT MOST OF THE DIVERSITY IN THE ANTI-HAPTEN RESPONSE

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Specificity is perhaps the most fundamental feature of the immune process. In this era of cellular and molecular immunology, much attention has been focused on the nature and distribution of immunoglobulin (Ig) cell surface receptors, but, relatively little attention has been directed to their precise functional activity. It is generally assumed that a precursor B-cell committed to a given antigenic determinant will be activated by appropriate receptor contact with that determinant giving rise to a clone of cells producing antibody molecules of like specificity. While there is little reason to doubt certain basic elements of such a proposal, there is good reason to question whether such a simple concept provides a comprehensive understanding of Ig receptor participation in the B-cell activation process.

Although immunization regularly elicits specific antibody, not all new Ig synthesized at the time of an immune response can be accounted for as specific antibody. Askonas and Humphrey observed in both guinea pigs and rabbits that half or more of newly synthesized Ig regularly failed to precipitate with immunogen (1). Moreover, Silverstein et al. have estimated that 95–99% of the new Ig produced by immunized fetal lambs often fails to react as specific antibody (2). Do these oft-ignored “nonspecific” Ig molecules represent the products of clones secreting antibody of particularly low affinity or avidity? Or do they instead represent the products of nonspecifically activated disparate clones secreting Ig irrelevant to the immunizing determinants?

Insight into these fundamental questions can perhaps best be obtained by studying newly synthesized Ig at the cellular level and by concentrating one's study on clones secreting molecules not normally detected as specific antibody. We have sought to do this using a variety of hapten-specific hemolytic plaque techniques some of which are introduced in this report. The basic experimental approach has been to immunize an animal with a single, chemically defined hapten and to subsequently assay its lymphoid organs not only for plaque-forming cells (PFC)¹ reactive with that hapten but also for PFC reactive with other haptens, many of which are structural analogues of the immunizing hapten.

Such an approach almost invariably reveals moderate to large numbers of PFC not previously detected in standard cellular studies of antibody formation.

¹ *Abbreviations used in this paper:* AGG, β -alanyl-glycylglycine; BSA, bovine serum albumin; DRBC, duck erythrocytes; HBSS, Hanks' balanced salt solution; PFC, plaque-forming cells; TGG, *N*-acetyl-L-tyrosyl-glycylglycine.

Many of these PFC are representatives of clones which expanded after immunization but which secrete molecules unable to lyse erythrocytes bearing the immunizing hapten. Thus, these hapten-activated clones represent antibody-secreting B cells which preferentially form plaques on sheep erythrocytes (SRBC) bearing structural analogues, but not on SRBC conjugated with the immunizing hapten. Multispecific plaque assay procedures and hapten inhibition techniques have provided useful information on the specificity and binding characteristics of the antibody molecules secreted by these analogue-reactive B-cell populations. The specificity data thus obtained allow initial insights into some of the complexities of Ig receptor participation in B-cell activation.

Materials and Methods

Animals. Female New Zealand white rabbits weighing from 2 to 3 kg were obtained from the NIH Animal Production Unit, Bethesda, Md. Female BALB/cN and C57BL/6N mice came from the same source and were used at 6-12 wk of age.

Reagents. SRBC were obtained from the NIH Animal Production Unit. Lyophilized guinea pig complement (C) was purchased from BioQuest Div. Becton, Dickinson & Co., Baltimore, Md. Fresh rabbit C was harvested from New Zealand white rabbits obtained from the NIH animal colony. It was held on ice and utilized in plaque assays within 2 h of acquisition. Ficoll (average mol wt 400,000) was obtained from Pharmacia Fine Chemicals, Inc., Piscataway, N. J.

Antigens. All the haptens employed in this study are listed in Table I. Large haptens have been given single capital letter designations; italicized lower case letters have been employed for the corresponding small haptens.² The procedures for synthesis of the special tripeptide-enlarged haptens were those reported by Inman et al. (3). Duck erythrocytes (DRBC) were obtained from Stanley B. Krutulis Co., Bridgeport, N. Y., and were derivatized with the large haptens, S, A, or Φ according to the method of Inman et al. (4). A₄₅-Ficoll³ was also prepared by a procedure described by Inman (5). DRBC were conjugated with the small haptens, *p*-azobenzenearsonate (*a*) and *p*-azobenzenesulfonate (*s*), as described by Hanna and Merchant (6). Rabbits immunized with hapten-derivatized DRBC ordinarily received 3 ml of 0.1% vol/vol hapten-derivatized DRBC stromata intravenously (i.v.) on days 0, 2, 4, 8, 15, and 30. Rabbits were rested thereafter, and those rabbits assayed at later intervals were normally boosted with the same dose of antigen only 3 days before sacrifice. Mice immunized with hapten-derivatized DRBC received 1.0 ml of 0.1% vol/vol of such DRBC intraperitoneally (i.p.) on day 0 and were assayed for splenic PFC on day 4. Mice immunized with haptened Ficoll received 10 μ g of A₄₅-Ficoll i.p. on day 0 and were assayed for splenic PFC on day 4.

Hemolytic Plaque Procedures. Immune cell suspensions were prepared as previously reported (6). For some experiments immune cell suspensions, frozen at 1°C/min and maintained in liquid N₂, were used after rapid thawing. The procedures involved have been published (7). Hapten-specific PFC were detected as previously described (8) except that the agar contained 0.2 mg bovine serum albumin (BSA) and 10³ U of sodium penicillin G/ml. All hemolytic plaque assays were performed without the use of base agar layers. Plates usually contained from 10 to 50 \times 10⁶ spleen cells. The plating, incubation, plaque development, and counting conditions were identical with those previously described (7). All data represent the SRBC background-corrected mean values for duplicate or triplicate hapten-specific PFC assays. A hyperimmune sheep anti-rabbit IgG serum was used for detection of rabbit indirect PFC. This antiserum had been raised in sheep after multiple monthly intramuscular injections of an emulsion containing 0.5 mg of Sephadex-purified rabbit IgG in 0.5 ml of 0.15 M NaCl and an equal volume of Freund's incomplete adjuvant. While enhancing or facilitating the detection of IgG-secreting PFC, this antiserum simultaneously inhibited the appearance of most IgM-secreting PFC. The average degree of IgM plaque inhibition was 70% with the antiserum diluted 1:100. Thus, the indirect hapten-specific PFC activities have been corrected both for background activity against SRC, and on an individual

² For letter designations corresponding to haptens see Table I.

³ An average of 45 molecules of hapten A per 400,000 daltons of original Ficoll were covalently bound to the aminoethylcarbonylmethyl (AECM₇₆) derivative (5).

basis for roughly 30% of the corresponding direct PFC activity which remained uninhibited by the facilitating antiserum.

Indicator Erythrocytes. Erythrocytes optimally derivatized with the large haptens were prepared according to the procedure of Inman et al. (4). This method normally attaches around 3×10^5 large haptenic groups to each erythrocyte. The method of Rouques et al. (7) was employed for the preparation of small hapten-conjugated indicator SRBC.

Multispecific PFC Assays. Detection in a single assay of two or more hapten-reactive PFC populations can be accomplished by the use of SRBC bearing optimal or near optimal densities of two or more haptenic determinants (7). In the present experiments, simultaneous diazonium coupling of SRBC with two different azo-haptens (e.g., *a* plus *s*) was accomplished by using, for each hapten, half the quantity of reagents required for single coupling and mixing equal volumes of the two separately prepared conjugation reagents just before the addition of the standard quantity of SRBC. If three, four, five, or six azo-haptens were coupled to indicator SRBC, then 1/3, 1/4, 1/5, or 1/6, respectively, of the quantity of reagents required for single hapten coupling were prepared for each hapten specificity. These were mixed in equal volumes just before addition of the standard quantity of SRBC required for the regular conjugation procedure as described by Rouques et al. (7). Variations in coupling rates for the different small azo-haptens probably prevented equivalent SRBC sensitization with each. Nonetheless the use of additional haptens in the coupling reaction regularly led to the detection of additional numbers of PFC.

Hapten-Mediated Inhibition of Hapten-Specific PFC. Sulfanilic acid (Fisher Scientific Co., Fair Lawn, N. J.) was diazotized and conjugated to BSA obtained from Miles-Pentex, Kankakee, Ill., by the method of Campbell et al. (9) to yield *s*₂₉-BSA. Arsanilic acid (Fisher Scientific Co.) was conjugated to BSA by the same method to yield *a*₂₆-BSA. DNP₃₂-BSA was prepared by the method of Eisen et al. (10). These hapten-derivatized proteins were dissolved in 0.15 M potassium phosphate buffer, pH 7.5, at a concentration of 10 mg/ml. Such multivalent inhibitors were added to the agar-bearing test tubes just before plating in order to yield a final concentration of 1 mg/ml of hapten-derivatized protein.

Hemagglutination Assays. All sera were inactivated at 56°C for 30 min. Veronal-buffered 0.15 M NaCl, pH 7.4 containing 1% normal rabbit serum was used for dilution of antisera. Antibody titrations were performed in disposable multi-well plastic plates (Cooke Laboratory Products Div., Dynatech Laboratories Inc., Alexandria, Va.) employing standard double-dilution procedures. The hemagglutination end point was defined as the reciprocal of the highest twofold dilution which provided visible agglutination after 2 h at 25°C.

Results

General Characteristics of Analogue-Reactive PFC. Initial experiments with hapten-specific hemolytic plaque assays demonstrated specificity characteristics similar to those expected. As experience accumulated, however, there were fairly frequent instances in which *a*-immunized rabbit spleen cell suspensions produced more PFC on *s*-coupled SRBC than on *a*-coupled SRBC, and conversely *s*-immunized rabbit spleen cell suspensions often produced more PFC on *a*-coupled SRBC than on *s*-coupled SRBC. In addition, spleen cell suspensions obtained from rabbits immunized repeatedly for weeks or months with a single azo-hapten would often produce indirect PFC responses specific for the immunizing hapten while their coexisting direct PFC responses would be predominantly directed against the alternate hapten. Such provocative findings could not be ignored.

In order to gain a more comprehensive insight into the functional specificity of PFC populations, various types of indicator SRBC were prepared, each bearing a different azobenzenoid hapten. Immune cell suspensions from rabbits immunized with a single azo-hapten were then examined in separate but parallel assays for the presence of PFC reactive with each of six or seven different azo-haptens. The azo-haptens chosen were selected to be either closely or distantly

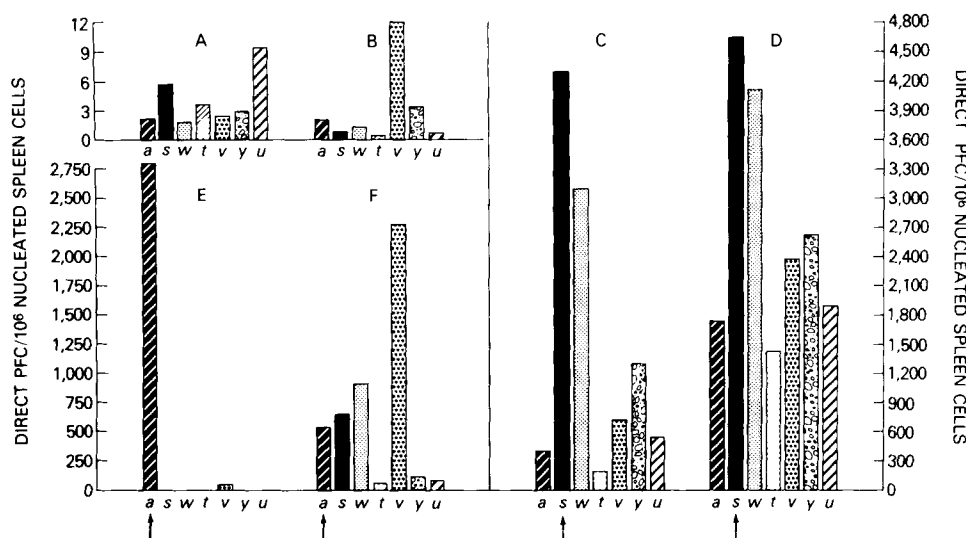


FIG. 1. Splenic direct hapten-specific PFC activities in azo-hapten immunized and control rabbits. Each histogram profile represents the activities detected in a single rabbit spleen. The arrows identify the hapten used for immunization. The haptens corresponding to the small letter designations are identified in Table I. Rabbit A was not immunized; B was assayed on day 33 after being immunized with 3 ml of 0.1% DRBC carrier only on days 0, 2, 4, 8, 15, and 30; C and D were each assayed on day 33 after being repeatedly immunized with 3 ml of 0.1% *s*-DRBC according to the same schedule; E and F were both immunized with *a*-DRBC and given six injections in the 1st mo as above. E was assayed 3 days after a boost at 28 mo and F was assayed 3 days after a boost at 8 mo.

related analogues of the arsonate- and/or sulfonate-immunizing haptens. Immunization with a single hapten usually elicited PFC populations detectable not only on SRBC bearing that hapten, but also on SRBC bearing both closely and distantly related haptenic structures.

The data assembled in Fig. 1 records findings that are typical for direct hapten-reactive PFC activities detectable in spleen cell suspensions of immunized and control rabbits. Each histogram bar represents the PFC activity obtained with indicator SRBC bearing a single azo-hapten. Although none of the haptens employed are known to exist in nature, spleen cells from normal, unimmunized adult animals invariably had background PFC activities against most or all assay haptens as depicted by rabbit A in Fig. 1. After correction for anti-SRBC activity, such background, hapten-specific PFC counts normally ranged from 2 to 8 per 10^6 nucleated spleen cells, as compared with background activities against SRBC which normally ranged from 1 to 5 per 10^6 cells. Immunization with a nonhaptented carrier antigen (DRBC), (rabbit B) produced at most, only minor fluctuations in these background, hapten-reactive direct PFC activities. In the great majority of instances, background hapten-specific, indirect PFC were either absent or so low as to escape detection, i.e., less than one indirect PFC per 10^6 nucleated cells.

The histograms for rabbits C and D are typical of the hapten-reactive, direct PFC responses of rabbits repeatedly immunized with *s*-DRBC. The histogram profiles for these two unrelated rabbits show marked similarities, and as might

be expected, the number of PFC detected on SRBC bearing the meta isomer of azobenzenesulfonate (*w*) is the second highest PFC activity detected. Typically also, the PFC activities observed for indicator SRBC bearing structurally distant analogues such as the *p*-azobenzophenone (*y*) and the *p*-bromophenylazo (*u*) haptens were also rather surprisingly high. The question that comes immediately to mind is whether the PFC detected on SRBC bearing hapten analogues represent subcomponents of the PFC population detected on *s*-SRBC or whether they represent separate and distinct PFC populations. It is also important to determine whether azo-hapten immunization invariably generates complex, diverse, hapten-reactive PFC responses or whether monospecific PFC responses are sometimes elicited.

That monospecific, direct PFC responses can occur which are detected only by erythrocytes bearing the immunizing hapten is apparent from the histogram for rabbit E which was rested for over 2 yr before a final booster immunization with *a*-DRBC. That all analogue-reactive PFC populations are not necessarily subcompartments of the specific PFC population is also apparent from the histogram for rabbit F which was assayed after a final *a*-DRBC boost at 8 mo. More PFC were seen on SRBC coupled with *s* or *w*, and nearly four times more on *p*-azobenzoate (*v*)-coupled SRBC than were seen on SRBC coupled with the immunizing hapten. In the occasional instances, of which rabbit F is typical, it is most unlikely that the *s*-, *w*-, or *v*-reactive PFC comprise subcompartments of the *a*-SRBC-reactive PFC population.

Evaluation of PFC Reactivity Directed to Common Structural Components Shared by Homologue- and Analogue-Coupled SRBC. The specificity of analogue-reactive, direct PFC populations as seen for the *v*-reactive PFC from rabbit F of Fig. 1 might be directed almost exclusively against "bridge components," common to all the haptenic structures employed, and comprising a phenylazo group attached to the subjacent amino acid components of the erythrocyte protein. However, these same structural features were present in the *t*-, *y*-, and *u*-SRBC which yielded only small numbers of plaques. In order to test further for the possibility of erythrocyte component contribution to reactivity, a series of enlarged azo-haptens were prepared (3). These haptens were designed to approximate the size of the average antibody-combining site, and they consisted of a tripeptide "stem" to which terminal azo-haptens were attached. When converted to reactive azides, these enlarged haptens coupled predominantly to epsilon amino groups of lysine residues present in erythrocyte surface proteins (4). Two separate series of enlarged haptens were prepared. One series shared a common acetyl-tyrosylglycylglycine (TGG) stem such that after conjugation it represented a haptened TGG-lysine determinant. The other series shared a β -alanyl-glycylglycine (AGG) stem such that after conjugation, its members represented haptened AGG-lysine determinants (see Table I). For the haptened TGG series, an unsubstituted acetyl-tyrosylglycylglycine hapten (G) served as a useful specificity control.

By employing both the small and the corresponding large azo-hapten indicator SRBC systems in parallel it was possible to evaluate the contribution of erythrocyte structures to PFC reactivity. If much of the PFC specificity was actually directed against erythrocyte-contributed components of the conjugation regions, (which conceivable might be shared by SRBC and DRBC) then the

TABLE I
Single-Letter Symbols and Chemical Designations* for Haptens
Employed in This Study

Small haptens		
<i>a</i>	=	<i>p</i> -azobenzeneearsonate
<i>s</i>	=	<i>p</i> -azobenzenesulfonate
<i>w</i>	=	<i>m</i> -azobenzenesulfonate
<i>o</i>	=	<i>o</i> -azobenzenesulfonate
<i>v</i>	=	<i>p</i> -azobenzoate
<i>y</i>	=	<i>p</i> -azobenzophenone
<i>u</i>	=	<i>p</i> -bromophenylazo
<i>t</i>	=	<i>p</i> -azobenzenesulfonamide
Large haptens		
TGG Series		
A	=	3-(<i>p</i> -arsonophenylazo)- <i>N</i> -acetyl-L-tyrosylglycylglycine
S	=	3-(<i>p</i> -sulfofenylazo)- " "
W	=	3-(<i>m</i> -sulfofenylazo)- " "
O	=	3-(<i>o</i> -sulfofenylazo)- " "
V	=	3-(<i>p</i> -carboxyphenylazo)- " "
Y	=	3-(<i>p</i> -benzoylphenylazo)- " "
U	=	3-(<i>p</i> -bromophenylazo)- " "
T	=	3-(<i>p</i> -sulfamylphenylazo)- " "
M	=	3-(<i>p</i> -trimethylaminophenylazo)- " "
Φ	=	3-phenylazo- " "
G	=	(3-unsubstituted)- " "
AGG Series		
J	=	<i>N</i> -(2,4-dinitrophenyl)-β-alanyl-glycylglycine
K	=	<i>N</i> -(2,4,6-trinitrophenyl)-β-alanyl-glycylglycine
D	=	<i>N</i> -(5-dimethylaminonaphthalene-1-sulfonyl)-β-alanyl-glycylglycine
Z	=	<i>N</i> -benzyloxycarbonyl-β-alanyl-glycylglycine

* Structural formulae for the large haptens are presented in reference 3. Each small hapten has the same structure as the substituted phenylazo portion of the large hapten designated with the corresponding upper case letter.

erythrocyte contribution should be substantially disrupted by the interposition of a tripeptide spacer. Fig. 2 presents typical direct and indirect hapten-reactive PFC profiles obtained for both small hapten-coupled and large hapten-coupled indicator SRBC from a rabbit immunized repeatedly for 1 mo with *s*-DRBC. The incidence of analogue-reactive PFC is not reduced by the use of large hapten PFC assay systems. On the contrary, each large hapten assay system consistently detected more PFC than the corresponding small hapten assay system. Since only very few PFC can be detected using the nonhaptened TGG tripeptide (G), it is apparent that most of the PFC antibody reactivities are directed against the distal or azo-hapten-defined termini of the large hapten molecules. If, however, the large PRC responses to W and A (Fig. 2) represented antibody directed against a phenylazotyrosyl "bridge," present as a portion of the small hapten-immunizing determinants, (and simply amplified in the large hapten assay system by a "spacing-out" effect), then the response to O, which shares this structure, should also have been large.⁴ Thus, it seems improbable that

⁴ Many additional examples of coexisting high and low PFC responses to different haptens sharing a phenylazotyrosyl bridge have been obtained and will be reported elsewhere. These results include instances in which a low response was detected with the unsubstituted phenylazo hapten, along with high responses to several of the other analogue haptens.

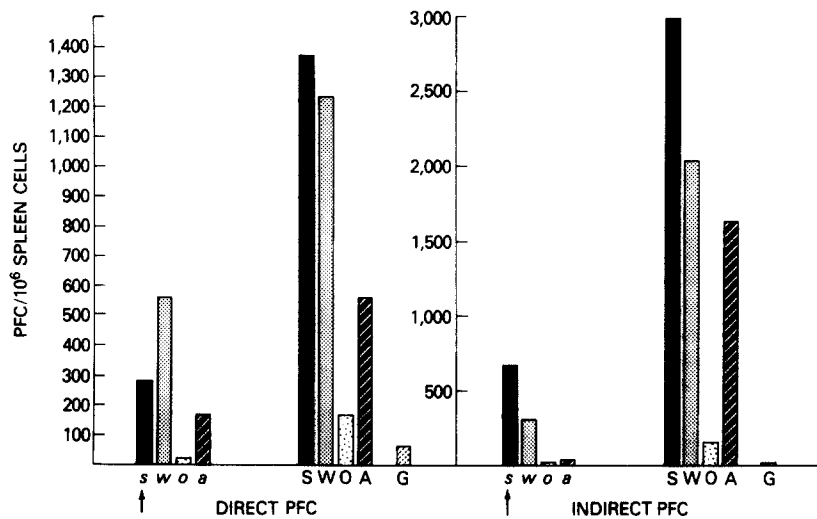


FIG. 2. Splenic PFC activities as discerned on small vs. large hapten-conjugated indicator SRBC. The various histograms represent the direct and indirect PFC activities detectable using either the large or the small hapten assay systems with the same rabbit spleen cell suspension. The haptens corresponding to the large and small letter designations are identified in Table I. This rabbit's spleen was assayed on day 33 after immunization with repeated i. v. injections of 3 ml of 0.1% *s*-DRBC on days 0, 2, 4, 8, 15, and 30.

analogue-reactive PFC populations are producing antibody that is exclusively directed against "bridge areas."

Analogue-Reactive PFC Responses in Mice. In addition to affording more reliable detection of hapten-reactive PFC populations, the increased sensitivity afforded by the large hapten assay systems had two other major effects. First, background, direct PFC activities increased on the order of 5- to 10-fold, thus permitting increased experimental inquiry into the nature and specificity of background, direct PFC populations. Some of this work has been reported elsewhere (11). Second, when used in conjunction with large hapten immunization, the large hapten assay systems allowed the ready detection of hapten-reactive PFC responses in mice, a capability possible for haptens like trinitrophenyl, but previously unattainable with small azo-hapten assay systems. Fig. 3 records the 4-day primary, direct PFC profiles of both BALB/c and C57BL/6 mice elicited in response to Φ -DRBC. The diversity of the PFC activities detected is obvious. The similarity in the histogram profiles between these two strains of mice differing at the major *H-2* histocompatibility locus is also striking. Immunization with a single, enlarged hapten has resulted in increased PFC activity for most of the enlarged haptenic specificities under test. Although a direct PFC population reactive with the immunizing hapten is invariably present in mice as in rabbits, the "specific" or homologue-reactive PFC population is by no means the most numerous single population detected. The Φ -reactive PFC were outnumbered in BALB/c mice by PFC reactive with three and in C57BL/6 mice by PFC reactive with four other haptenic determinants. Although the S, A, and V haptens can be considered close analogues which share the stem TGG component with the immunizing Φ -hapten, the enlarged

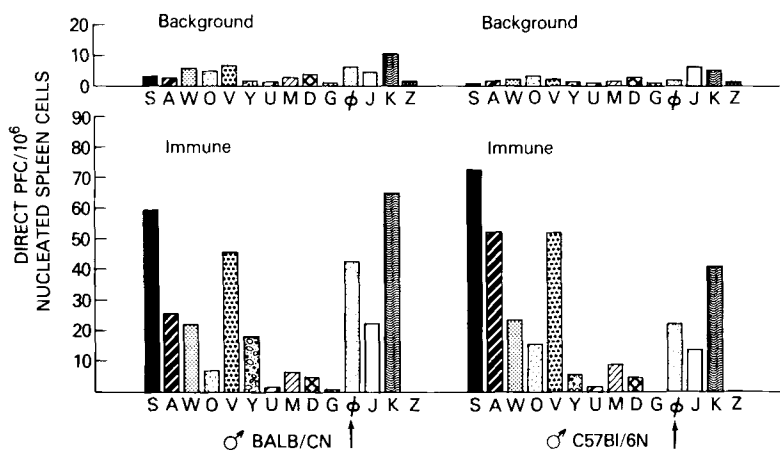


FIG. 3. Direct, hapten-specific PFC activities in mice immunized with Φ -DRBC. Each histogram represents the activities detected in a cell suspension prepared from a pool of seven mouse spleens. Each bar represents the mean of duplicate assays for a given haptenic specificity after correction for background PFC against SRBC. BALB/cN and C57Bl/6N mice were immunized i.p. with a 1.0 ml of 0.1% Φ -DRBC on day 0 and assayed on day 4. Unimmunized control mice were assayed on the same day and their SRBC-corrected splenic PFC activities are shown as background histograms.

trinitrophenyl hapten, K, must be considered less closely related not only at its terminus, but also because it contains an AGG rather than a TGG "stem" component. PFC against other relatively disparate structures such as the trimethylaminophenylazo-TGG (M) and the dansyl-AGG (D) haptens also often show detectable increases after Φ -DRBC immunization.

Are Analogue-Reactive PFC Representative of Hapten-Elicited Humoral Immune Responses? Consistent findings such as these served to raise the question whether antibody activities measured as PFC might reflect some spurious laboratory artifact unique to hemolytic plaque assay systems. To investigate this possibility, rabbits were immunized repeatedly with Φ -DRBC, and their splenic PFC and serum hemagglutinin titers were compared on day 18. Φ -DRBC-immunized rabbits are often uniquely slow in converting to IgG production. At this stage, these rabbits were still committed exclusively to IgM responses, and no indirect PFC were detectable. Serum obtained from these rabbits at sacrifice was assayed by passive hemagglutination on large hapten-conjugated SRBC. With only occasional exceptions, hapten-reactive PFC levels correlated well with serum titers (Fig. 4). In all instances, the hapten-reactive PFC responses were extremely diverse. In rabbit A both the PFC and serum titer activities against analogue haptens often exceeded the activities demonstrable on SRBC bearing the immunizing hapten, Φ .

As in mice, the rabbit PFC activities against more disparate haptens such as those PFC reactive with the Y, D, J, and K haptens substantially exceeded background, direct PFC activities. (Background PFC activities detected on large hapten-coupled SRBC usually range from 1 to 20 per 10^6 spleen cells in most unimmunized rabbits. The hapten-specific hemagglutination titers for preimmune bleedings from rabbits A and B ranged from <2 to 4.) Thus, immunization

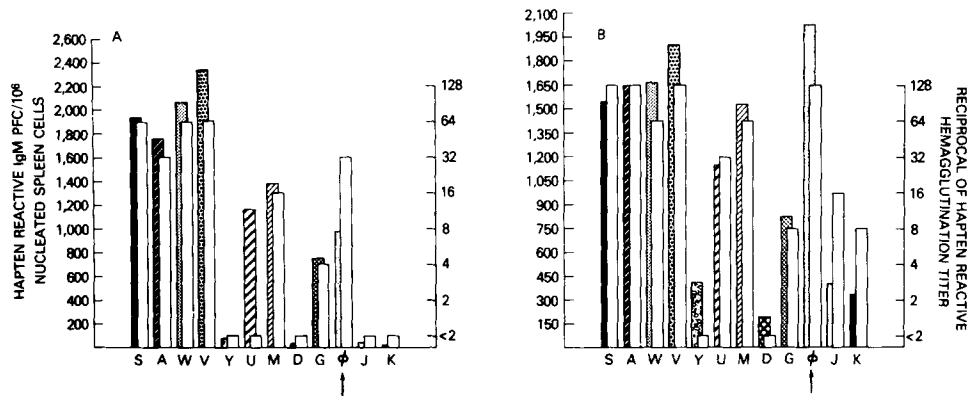


Fig. 4. General correlation between splenic, direct PFC values, and serum hemagglutination titers. The shaded histograms, A and B, represent the PFC activities detected in separate rabbit spleens. Each shaded bar represents the mean of triplicate assays for a given haptenic specificity after correction for background PFC against SRBC. Each open bar represents the reciprocal of the hemagglutination titer obtained for a given haptenic specificity. Rabbits A and B were each immunized with repeated i.v. injections of 3 ml of 0.1% Φ -DRBC on days 0, 2, 4, 8, and 15 and their spleens were assayed on day 18. Blood for serum hemagglutination titers was drawn about 5 min before acquisition of the spleens.

of rabbits with a single, large haptenic determinant regularly generated increased PFC and hemagglutinating activities against most of the enlarged haptenic determinants tested. Such findings indicate that the diverse analogue-reactive PFC detected are the source of diverse humoral antibodies formed in response to immunization.

It is also relevant to inquire whether some vagary in C binding attributable to the heterologous activation of guinea pig C by rabbit Ig might impose a misleading impression of PFC response diversity. Accordingly, PFC from Φ -immunized rabbits were compared using either lyophilized guinea pig serum or fresh rabbit serum as separate sources of C. A typical result is illustrated in Fig. 5. Appreciable variations in response specificity were encountered using the separate C sources; however, guinea pig C appears to constitute, if anything, a more conservative system for the evaluation of PFC response diversity.

Are Analogue-Reactive and Homologue-Reactive PFC Cross-Reacting or Mutually Exclusive? Since analogue-reactive and homologue-reactive PFC were detected on separate assay plates, it was important to ascertain whether they represented largely overlapping "cross-reactive" PFC populations, or whether they represented coexisting but mutually exclusive PFC responses. There are several potential experimental approaches to this question.⁵ For the present some early plaque inhibition experiments utilizing the small azo-hapten assay systems are given below. Table II records the results of a typical experiment performed with direct hapten-reactive PFC from a rabbit immunized repeatedly with s-DRBC. Direct PFC reactive with the immunizing hapten s were the largest single hapten-reactive plaque population detected; a- and t-reactive PFC were also numerous. When combinations of two or more haptenic analogues

⁵ Merchant, B., and J. K. Inman. Manuscript in preparation.

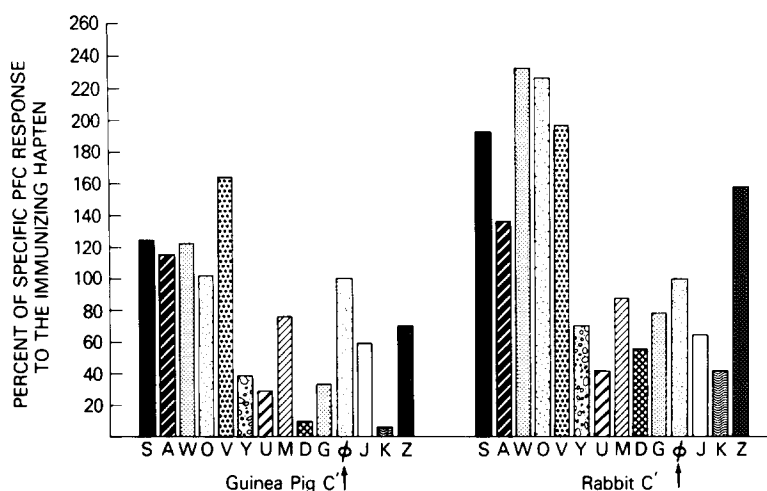


FIG. 5. Detection of direct hapten-specific rabbit PFC with either rabbit or guinea pig C. Both histograms represent the PFC activities detected using the same rabbit spleen cell suspension. This rabbit had been immunized i.v. with 3.0 ml of 0.1% Φ -DRBC on days 0, 2, 4, 8, and 15, and its spleen cell suspension was assayed on day 18. Like rabbits A and B of Fig. 4, at this juncture it had no detectable indirect PFC. The data have been normalized and are presented as the percent of the specific PFC response to Φ , the immunizing hapten. The hemolytic efficiency of lyophilized guinea pig C was greater than that of fresh rabbit C. Guinea pig C detected 977 anti- Φ PFC per 10^6 nucleated spleen cells while rabbit C detected only 439 anti- Φ PFC per 10^6 nucleated spleen cells.

were coupled to SRBC, additive (but less than fully summative) PFC activities were detected. When these PFC were exposed to equal concentrations of multivalent haptened BSA inhibitors variable numbers of PFC were inhibited. In all instances, BSA alone or DNP-BSA had little inhibitory effect. Sulfonate-reactive PFC were well inhibited by s_{29} -BSA and arsonate-reactive PFC were well inhibited by a_{26} -BSA. However, a_{26} -BSA was, at best, a weak inhibitor of s -reactive PFC and s_{29} -BSA was a weak inhibitor of a -reactive PFC. Thus, the reactivity of a given analogue-reactive PFC population can be higher for the analogue test hapten than for the original immunizing or homologue hapten. The t -reactive PFC were also moderately well inhibited by a_{26} -BSA but were not inhibited by s_{29} -BSA.

With multi-haptened indicator SRBC, s_{29} -BSA was often a better inhibitor than a_{26} -BSA but it became a progressively less effective inhibitor as additional haptens were added to the indicator SRBC. Multi-haptened SRBC bearing six different azo-haptens provided detection of approximately twice as many PFC as could be detected by SRBC bearing just the immunizing homologue s . Only about half of these PFC could be inhibited by s_{29} -BSA, and a_{26} -BSA was a comparably effective inhibitor.

Table III reports a similar experiment performed with the direct PFC obtained from a rabbit immunized repeatedly with a -DRBC. In this instance, more PFC were detectable on s -conjugated SRBC than on SRBC bearing the immunizing homologue, a . Again, conjugation of both these haptens to indicator SRBC led to additive (but less than fully summative) numbers of direct PFC. Conjugation of

TABLE II
*Hapten Inhibition Studies of Direct Analogue-Reactive PFC from a s-DRBC Immune Rabbit**

Indicator red cells‡	No. of hapten-reactive PFC/10 ⁶ spleen cells§	Inhibiting antigen (1 mg/ml)	% of PFC inhibition
<i>s</i> -SRBC	2,402	None	—
"		BSA	8
"		DNP ₃₂ BSA	0
"		<i>a</i> ₂₆ BSA	17
"		<i>s</i> ₂₉ BSA	94
<i>a</i> -SRBC	913	None	—
"		BSA	0
"		DNP ₃₂ BSA	0
"		<i>a</i> ₂₆ BSA	100
"		<i>s</i> ₂₉ BSA	9
<i>t</i> -SRBC	1,994	None	—
"		BSA	16
"		DNP ₃₂ BSA	0
"		<i>a</i> ₂₆ BSA	65
"		<i>s</i> ₂₉ BSA	0
<i>a</i> -SRBC- <i>s</i>	2,930	None	—
"		BSA	1
"		DNP ₃₂ BSA	0
"		<i>a</i> ₂₆ BSA	73
"		<i>s</i> ₂₉ BSA	76
<i>s o</i> SRBC	4,372	None	—
"		BSA	0
<i>w t</i> SRBC		DNP ₃₂ BSA	10
"		<i>a</i> ₂₆ BSA	72
"		<i>s</i> ₂₉ BSA	53
<i>s v o</i> SRBC	4,924	None	—
"		BSA	5
<i>w y t</i> SRBC		DNP ₃₂ BSA	0
"		<i>a</i> ₂₆ BSA	42
"		<i>s</i> ₂₉ BSA	47

* This rabbit's spleen was assayed on day 33 after being immunized i. v. with 3.0 ml of 0.1% *s*-DRBC on days 0, 2, 4, 8, 15, and 30.

‡ The haptens corresponding to the small letter designations are identified in Table I. Indicator SRBC shown in association with two or more letters were prepared by exposure to equimolar concentrations of the reactive form of two or more haptens and were employed for multispecific PFC assays which allow the simultaneous detection of two or more PFC populations in a single assay.

§ Each value represents the mean of triplicate assays after correction for background PFC activity against SRBC.

|| For the four types of indicator red cell preparations bearing the *s* hapten, the computed numbers of plaques inhibited by *s*₂₉BSA were 2,258, 2,227, 2,317, and 2,314 for *s*-SRBC through *s v o w y t*-SRBC, respectively.

TABLE III
Hapten Inhibition Studies of Direct Analogue-Reactive PFC from an *a*-DRBC Immune Rabbit*

Indicator red cells‡	No. of hapten-reactive PFC/10 ⁶ spleen cells§	Inhibiting antigen (1 mg/ml)	% of PFC inhibition
<i>a</i> -SRBC	1,253	None	—
"		BSA	13
"		DNP ₃₂ BSA	15
"		<i>a</i> ₂₆ BSA	100
"		<i>s</i> ₂₉ BSA	42
"		<i>a</i> ₂₆ BSA + <i>s</i> ₂₉ BSA	100
<i>s</i> -SRBC	1,541	None	—
"		BSA	7
"		DNP ₃₂ BSA	7
"		<i>a</i> ₂₆ BSA	0
"		<i>s</i> ₂₉ BSA	79
"		<i>a</i> ₂₆ BSA + <i>s</i> ₂₉ BSA	83
<i>w</i> -SRBC	990	None	—
"		BSA	9
"		DNP ₃₂ BSA	10
"		<i>a</i> ₂₆ BSA	60
"		<i>s</i> ₂₉ BSA	41
"		<i>a</i> ₂₆ BSA + <i>s</i> ₂₉ BSA	62
<i>a</i> -SRBC- <i>s</i>	2,356	None	—
"		BSA	8
"		DNP ₃₂ BSA	15
"		<i>a</i> ₂₆ BSA	79
"		<i>s</i> ₂₉ BSA	52
"		<i>a</i> ₂₆ BSA + <i>s</i> ₂₉ BSA	100
<i>s w</i> SRBC	3,884	None	—
"		BSA	19
<i>a t o</i>		DNP ₃₂ BSA	14
"		<i>a</i> ₂₆ BSA	18
"		<i>s</i> ₂₉ BSA	59
"		<i>a</i> ₂₆ BSA + <i>s</i> ₂₉ BSA	53

* The rabbit's spleen was assayed on day 33 after being immunized i. v. with 3.0 ml of 0.1% *a*-DRBC on days 0, 2, 4, 8, 15, and 30.

‡ The haptens corresponding to the small letter designations are identified in Table I. Indicator SRBC shown in association with two or more small letters were exposed to conjugation with equimolar concentrations of two or more haptens and were employed for multispecific PFC assays which allow the simultaneous detection of two or more PFC populations in a single assay.

§ Each value represents the mean of triplicate assays after correction for background PFC activity against SRBC.

|| Each present at 1 mg/ml as an inhibitor.

five different azo-haptens to indicator SRBC led to detection of more than three times as many PFC as could be detected on SRBC bearing only the *a* homologue. Only modest numbers of PFC were inhibited by exposure to 1 mg/ml of BSA or DNP₃₂-BSA. Arsonated BSA was fully effective in inhibiting plaques detectable

on *a*-SRBC, but it was quite ineffective in suppressing plaques detectable on *s*-SRBC. The *s*-reactive PFC could be well inhibited however by *s*₂₉-BSA. When indicator SRBC were conjugated with both the *a* and *s* haptens, *s*₂₉-BSA could only inhibit about half of the PFC detected. When both *a*₂₆-BSA and *s*₂₉-BSA were added as inhibitors, all plaques could be inhibited. SRBC coupled with each of five analogue azo-haptens could be only partially inhibited by *s*₂₉-BSA and this inhibition was not increased by the concurrent addition of *a*₂₆-BSA.

Possible T-Cell Participation in the Diversity of PFC Responses. The diversity of PFC responses engendered by hapten immunization might be attributable strictly to thymic-dependent antigens and to the consequent complexities afforded by T-cell participation in the humoral response. Accordingly, mice were immunized with the enlarged arsonate hapten, A, coupled either to the thymic-dependent carrier, DRBC, or to the thymic-independent carrier, Ficoll. Fig. 6 discloses the resulting 4-day primary, direct PFC profiles elicited in BALB/c mice. Although these PFC profiles show some differences, they are nonetheless unquestionably diverse in both instances. Thus, the direct PFC response to azo-haptens appears to be diverse regardless of whether thymic-dependent or thymic-independent carrier antigens are employed.

Discussion

Our present data (Tables II and III) are incompatible with the view that analogue hapten assay systems detect only subcomponents of the PFC population detected on SRBC bearing the immunizing hapten. Instead, they favor the view that immunization with a single, chemically defined azo-hapten elicits numerous coexisting PFC populations, each best detected either with the homologue hapten or with one or another hapten analogue conjugated to indicator SRBC. The data are however, consistent with the view that some overlapping occurs in the detection of these coexistent PFC populations since partially additive rather than fully summative detection of two separate PFC response specificities is usually observed when SRBC conjugated with two or more separate azo-haptens are employed as the indicator system.

Our present findings also indicate that the usual hapten-reactive PFC assay may be "seeing" only the tip of the humoral immune response iceberg. The use of analogue haptens for PFC assays reveals additional, largely nonoverlapping PFC populations, a circumstance analogous perhaps to "rolling over" the iceberg in such a way as to expose new tips. Although numerous hapten analogues may be employed to view numerous tips of the immune response iceberg, a full populational definition of the totality of the secretory B-cell response remains elusive. Since early direct PFC specific for the immunizing hapten often represent only a very minor component even of the selected populations tested for, it follows that a full comprehension of the functional specificity of Ig receptor-mediated participation in B-cell activation also remains elusive. Thus, when only the immunizing or homologue hapten is employed for PFC assays a question may often fairly be raised as to the actual representativeness of the PFC population detected. An important corollary of the foregoing is that the usual homologue-reactive PFC population probably represents a restricted number of clones. Up to now, PFC populations have been viewed as having restricted

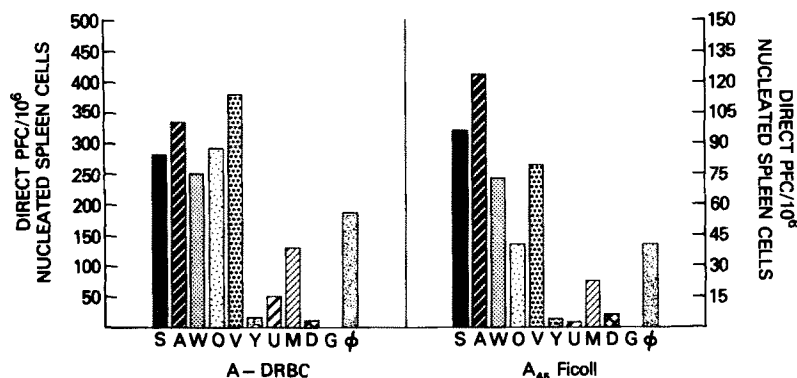


FIG. 6. Hapten-specific, splenic PFC responses of BALB/cN mice to thymic-dependent and thymic-independent antigens. Each histogram represents the direct PFC activities detected in a cell suspension prepared from a pool of six mouse spleens. Each vertical bar represents the mean of duplicate assays for a given haptenic specificity after correction for background PFC against SRBC. BALB/cN mice were immunized i.p. either with 1.0 ml of 0.1% A-DRBC or with 10 μ g of A₄₅-Ficoll on day 0 and their spleens were assayed on day 4.

heterogeneity only under special circumstances of immunization (12-14). The present findings indicate that most secretory B-cell clones detectable as hapten-reactive PFC constitute more or less restricted subcomponents of the total secretory B-cell response.

One might envisage similar circumstances of PFC detection if all hapten-specific assays were inherently inadequate; i.e., if by their very nature, all hemolytic plaque techniques were of low sensitivity. Although PFC assays employing small azo-haptens may suffer to some extent from this limitation, PFC procedures which use the enlarged azo-haptens usually have markedly increased sensitivity, even under circumstances in which immunization has been conducted with a small azo-hapten (Fig. 2). In separate studies, the large hapten assay systems have consistently detected from 90 to 98% of MOPC 315 myeloma tumor cells as plaque formers (15). Since the large haptens all couple to SRBC by a common coupling reaction (4) it is also likely that the sensitivities for each of the large hapten assay systems are closely comparable. Thus, it appears that the enlarged hapten assay systems succeed in detecting nearly all of any given tip on the immune response iceberg.

Since the PFC populations detected by the various hapten-reactive plaque assays are mostly separate and nonoverlapping (Tables II and III), the analogue-reactive PFC account, in all probability, for some of the "nonspecific" new Ig produced in response to immunization (1, 2). Although much of this new Ig appears to have comparable specificities in both hemagglutination and hemolysis reactions (Fig. 4), it is quite conceivable that comparison by other less closely related immunologic procedures might reveal a greater divergence of reactive specificities. In other words, many of the newly synthesized Ig molecules secreted by analogue-reactive PFC might be perceived as "nonspecific" new Ig in other types of immunologic assays.

Previous studies have established the existence of "heteroclitic" antibodies (16, 17). These heteroclitic antibodies are generally defined as having a higher

TABLE IV
Classification of Hapten-Elicited PFC on the Basis of Homologue and Analogue Plaque-Forming Reactivity

Characteristics of Ig-secreting cells in hapten-reactive plaque formation*	Traditional terminology	Suggested terminology
I. Homologue-reactive, Analogue-nonreactive	"Monospecific"	Homoreactive
II. Homologue-reactive, Analogue-reactive	Cross-reactive	Cross-reactive
III. Homologue-nonreactive, Analogue-nonreactive	"Nonspecific"	Nonreactive
IV. Homologue-nonreactive, Analogue-reactive	(Unrecognized)	Alloreactive

* In any hapten-elicited immune response, the recognizable size of each PFC compartment is determined operationally, i.e., largely by the number and sensitivity of the haptenic assay systems employed in screening for Ig-secreting cells. For example, as the number of assay systems is increased compartment IV tends to enlarge at the expense of compartment III.

binding affinity for a cross-reacting determinant than for epitopes of the original immunizing antigen. Heteroclitic antibodies have usually been detected only in limited instances (18, 19) and unlike the antibodies secreted by analogue-reactive PFC, heteroclitic antibodies have usually been defined in terms of their affinity rather than their functional reactivity.

Analogue-reactive PFC are secreting Ig molecules which do not readily seem to fit the usual definitions for cross-reactive, for nonspecific, or even for heteroclitic Ig molecules. Since hapten-reactive PFC can be conveniently defined in terms of their detectability on SRBC bearing either haptenic homologues or analogues, these relationships are set forth systematically in Table IV. Existing concepts include monospecific, cross-reactive, and nonspecific PFC, however the present findings suggest an additional category of Ig-secreting cells which might best be termed alloreactive, i.e., they are preferentially reactive in plaque formation with alternative or analogue structures other than the eliciting hapten. Since analogue-reactive or alloreactive PFC responses are generally more numerous for specificities that represent closely related rather than distantly related haptenic analogues (Figs. 1-4 and 6) they are distinct from polyclonal, PFC responses which are usually of much lower amplitude and are not subject to recognizable specificity requirements (20-22).

Alloreactive PFC populations have not been previously reported. Perhaps this is mainly because they have not been systematically sought. Alloreactive PFC populations exist in both direct (IgM) and indirect (IgG) immune responses (Fig. 2). They are detectable by both traditional small hapten and enlarged spacer hapten assay systems, and they do not appear to be generally referable to antibody activities directed against bridge, spacer, or stem components of haptenic structures. Such alloreactive PFC comprise a large component of direct (especially early direct) PFC responses. It is not uncommon for a single alloreactive PFC population to outnumber the population of PFC detectable on SRBC bearing the actual immunizing hapten (Figs. 1 F, 3, and 4 A). Alloreactive PFC appear in response to both thymic-dependent and thymic-independent antigens (Fig. 6). And, they often comprise the major portion of hapten-elicited B-cell immune responses both in rabbits and in mice (Figs. 1 F and 3).

Most B cells which have matured to the stage of active Ig secretion, are committed to the synthesis of antibody molecules which are homogeneous in

reactive specificity (23-25). In addition, secretory B cells rarely if ever undergo capricious changes in specificity once secretion has commenced (6, 25, 26). Thus, the usual mature B cell appears to be highly differentiated and "locked-in" to the production of a given pair of V-region structures. Numerous biosynthetic constraints also appear to be imposed upon the immediate precursors of secretory B cells (27-29). It is generally assumed that a precursor B cell bearing Ig receptors committed to a given antigenic determinant will, on activation, give rise to a clone of cells producing antibody molecules of like specificity. Considerable experimental evidence supports this view (30, 31). Thus there appear to be few if any "defections" of the Ig specificity commitments of individual B cells from their precursor to their secretory stages. If these concepts are viewed as axiomatic, then the very existence of alloreactive PFC challenges any simplistic interpretation of Ig receptor function in the B-cell activation process. Ig receptors may be viewed as playing either an active (32, 33) or a passive (22) role in B-cell activation. In either instance they are considered to play a specific or highly selective role. Our present findings suggest the existence of a substantial degree of cross-activation affecting B-cell clones having favorable reactivities with analogues of the immunizing hapten. Hapten inhibition studies of alloreactive PFC populations, should provide much useful information on the affinity and the avidity characteristics of the Ig molecules they secrete. Systematic studies of alloreactive PFC may ultimately provide fundamental insights into the complex process by which B precursor cells are activated to secretory function. Such studies may also yield useful insights into the nature of Ig receptor participation in B-cell activation.

Summary

Immunization of rabbits or mice with a single, chemically defined hapten elicits populations of plaque-forming cells (PFC) detectable not only on sheep erythrocytes (SRBC) bearing the immunizing hapten, but also on SRBC bearing structural analogues of the immunizing hapten. Most of these analogue-reactive PFC preferentially lyse analogue-conjugated SRBC and cannot be detected on erythrocytes bearing the immunizing hapten. Thus, they represent heretofore largely unstudied components of the secretory B-cell response to haptenic immunization, and they have been termed alloreactive PFC.

Such alloreactive PFC are detectable using either classical small haptens or tripeptide-enlarged counterparts of these classical haptens. They are present in large numbers both in direct and in indirect PFC assays, and they are elicited in response to both thymic-dependent and thymic-independent antigens. Relatively few alloreactive PFC can be attributed to cells producing hapten-carrier or "bridge area"-specific antibodies. Since the antibodies released by alloreactive PFC can also be detected by passive hemagglutination, their presence does not appear attributable to vagaries of complement activation.

Numerous coexisting alloreactive PFC populations are detectable after haptenic immunization. In early direct PFC responses it is not uncommon for a single alloreactive PFC population to outnumber the population of PFC detectable on SRBC bearing the actual immunizing hapten. These alloreactive PFC may be the source of at least some of the new "nonspecific" Ig which is formed at

the time of immunization but about which little is known for lack of available techniques. Some possible implications of these findings on the specificity of B precursor cell activation are discussed.

Received for publication 16 September 1976.

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