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Antibodies with idiotypic and anti-idiotypic reactivity (epibodies) in conventional immune responses to dinitrophenylated carriers

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SUMMARY

A number of monoclonal antibodies were derived from the spleen cells of dinitropheryl (DNP)-immunized mice. Both T-dependent and T-independent carriers were used, and the intensity and length of immunization were varied. It was found that some of the antibodies had only idiotypic (Ab1) reactivity, while others had both idiotypic (Ab1) and anti-idiotypic (Ab2) reactivity. Among the latter antibodies some molecules reacted specifically with DNP and with the combining site of anti-DNP antibodies (epibodies), while others bound DNP and anti-DNP Abs as well as a variety of unrelated antigens (polyreactive antibodies). The proportion of the three types of antibodies (antigen-specific, epibodies and polyreactive antibodies) varied with the nature of the carrier, the intensity of the immunization, and the length of the immunization process. Further characterization of the epibodies, which were predominant in the secondary response to DNP-keyhole limpet haemocyanin (KLH), showed that both Ab1 and Ab2 reactivities were inhibited by both soluble ligands (DNP and anti-DNP), indicating that the specific combining site of the monoclonal antibodies (mAbs) (and/or of the rabbit anti-DNP antibody in the case of Ab2) was involved in both activities. Both Ab1 and Ab2 reactivities were removed by absorption of the mAbs with either immobilized DNP or immobilized rabbit anti-DNP. The mAbs were capable of binding themselves as well as to other mAbs with the same characteristics. The affinity constants of several mAbs for both the DNP and anti-DNP ligands were determined.

INTRODUCTION

In a previous report, we investigated the kinetics of idiotypic (Ab1) and anti-idiotypic (Ab2) anti-dinitrophenyl (DNP) antibodies elicited in mice immunized with dinitrophenylated carriers.[1] We found that the kinetics and magnitude of both antibody responses, following immunization with the T-independent antigen DNP-Ficoll, were very similar. Three possibilities may account for this finding. First, there may have been distinct and independent, but parallel, Ab1 and Ab2 responses. Second, there could have been antibody molecules carrying both anti-hapten (Ab1) and anti-anti-hapten (Ab2) reactivities. Such antibodies have been described and designated epibodies, or Ab2ε.[2–4] They have been reported particularly in association with autoimmune conditions such as rheumatoid arthritis,[5] systemic lupus erythematosus[6,7] and myasthenia gravis.[8] Their presence has been suggested, but not proven, in a conventional immune response.[9] As a third alternative, the observed antibody responses could be caused by polyreactive antibodies which bind, with low affinity, both the eliciting antigen as well as a wide range of self-antigens, including self-idiotypes. Polyreactive antibodies have also been described in autoimmune conditions,[10,11] and in association with $CD5^+$ (Ly-1) B lymphocytes[12] in normal spleens and in spleens of mice injected with lipopolysaccharide (LPS).[13,14]

In order to distinguish between these possibilities, a number of monoclonal antibodies (mAbs) from DNP-immune mouse spleens were produced. DNP conjugated to different carriers was used as an immunogen with varied immunization regimens. The mAbs were examined for their ability to bind, in addition to DNP, anti-DNP antibodies of rabbit origin as well as a panel of antigens commonly used to detect polyreactivity. A number of monoclonal epibodies were characterized in greater detail, paying particular attention to their specificity. They were titrated against each of their ligands (DNP and anti-DNP) and their affinity constant for each ligand was measured. The ability of each ligand to inhibit the binding of the monoclonal antibodies to wells coated with either DNP-bovine serum albumin (BSA) or rabbit anti-DNP was investigated as well as the ability of the monoclonal epibodies to bind to themselves.

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†Diego Segre passed away in August 1996, after a long illness. his appreciation of the Network Theory greatly influenced the early stages of this research.

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MATERIALS AND METHODS

Animals

Female BALB/c mice (2–6-months old) were obtained from Harlan-Sprague-Dawley (Indianapolis, IN) for use as fusion donors.

Immunization of fusion donors

Mice receiving a primary immunization were injected intraperitoneally (i.p.) with 0·2 ml of a 500 μg/ml solution of DNP_{60}-Ficoll, or 0·1 ml of a 4% suspension of DNP-Ba (*Brucella abortus*), or with 0·5 ml of a 200 μg/ml suspension of DNP_{14}-KLH (keyhole limpet haemocyanin substituted with 14 DNP groups per 100 000 daltons of protein),[15] adsorbed on bentonite.[16] Fusions were performed on the 4th or 5th day after the injection. Secondary immunizations with DNP-KLH were given intravenously (i.v.) at varied time intervals following the primary immunization (3 weeks or 11 weeks), using 100 μg of soluble antigen. Three days after the secondary immunization the spleens were taken and fused. Mice receiving multiple immunizations were injected i.p. with 100 μg DNP-KLH on bentonite, twice at 3-week intervals, and a third time i.v. with 100 μg of soluble antigen 3 days before fusion.

Affinity-purified antibodies

Rabbit affinity-purified antibodies, O anti-DNP, anti-ovalbumin (OVA), and anti-chicken immunoglobulin G (IgG), were prepared in our laboratory by adsorption to a column of insolubilized antigen followed by elution with either the specific hapten or by lowering the pH to 3·5.[1,17]

Fusion

Hybridoma lines were produced by fusion of SP2/0 plasmacytoma cells and spleen cells from immunized mice following the procedure of Oi & Herzenberg.[18] After fusion, the pellet was gently resuspended with Dulbecco's modified Eagle's minimal essential medium-HAT (DMEM-HAT) with 15% horse or calf serum and distributed at decreasing cell concentrations in several flat-bottom 96-well plates; a comparable number of unfused, immune spleen cells was plated in several wells of each plate. The medium was changed every 3 to 4 days. Within 3 weeks, the supernatants of the wells (referred to as culture supernatants) were tested by enzyme-linked immunosorbent assay (ELISA). Positive wells were retested and expanded, and the cultures cloned and frozen in liquid nitrogen. Over 25 fusions were obtained with spleens of mice immunized with DNP conjugated to different carriers (DNP-Ficoll, DNP-Ba, DNP-KLH primary, DNP-KLH secondary).

Clones

Over 200 positive cultures obtained from a large portion of the fusions of SP2/0 plasmacytoma cells and anti-DNP immunized spleen cells were cloned once or twice by limiting dilution. Supernatants (referred to as mAb) were obtained from spent cultures and filtered through 0·22 μm filters.

Purified monoclonal antibodies. MAbs produced in cultures were passed through a goat anti-mouse immunoglobulin (H + L)-Sepharose 4B column, eluted with acid buffer (pH 5·5, 4·3 or 2·3), dialysed against borate-buffered saline (BBS), pH 8·3, and concentrated on an Amicon filter apparatus. MAbs produced in ascites fluid were purified as described by Reik *et al.*[19]

Preparation of $F(ab')_2$ and Fab fragments. MAbs were digested with pepsin or papain, following the techniques of Andrew & Titus.[20]

Biotinylation. MAbs at a concentration between 0·5 and 0·75 mg/ml were mixed with biotinamide caproate N-HydroxySuccinimide (NHS) esther in HEPES buffer pH 7·5 at a molar ratio of 1:40, left at room temperature for 90 min, passed through a Bio-Gel P-6DG (Bio-Rad Laboratories, Richmond, CA) column in phosphate-buffered saline (PBS), and concentrated.

Identification by ELISA of idiotypic (Ab1 reactivity), anti-idiotypic (Ab2 reactivity) and polyreactive antibodies

Ab1 reactivity was tested on ELISA plates coated with DNP-BSA. Ab2 reactivity was routinely tested on plates coated with rabbit affinity-purified anti-DNP antibodies or their $F(ab')_2$ fragment; in some experiments, plates were coated with the $F(ab')_2$ or the Fab fragments of two mouse mAbs that showed strict specificity for DNP only. Polyreactivity was tested on plates coated with a panel of several different antigens. Flat bottom 96-well plates (Immulon 2 or Immulon 4 strips, Dynatech, Chantilly, VA) were coated overnight with 10 μg/ml coating reagent in carbonate buffer (pH 9·5), blocked overnight with either egg white buffer[21] or 1% BSA in PBS and washed with PBS-Tween 20. Fifty microlitres of culture supernatant was added and the plates were incubated for 90 min at room temperature. After repeated washings, bound immunoglobulin was detected by the addition of 50 μl of β-galactosidase (β-gal) (Zymed Laboratories Inc., South San Francisco, CA) or horseradish peroxidase (HRPO)-labelled goat anti-mouse (H + L) immunoglobulin (Southern Biotechnology Associates, Birmingham, AL). When plates were coated with $F(ab')_2$ or FAb of mouse anti-DNP mAb, HRPO-labelled protein A or protein G was used. After incubation for 90 min and repeated washings, 100 μl/well of the appropriate substrate, either *o*-nitrophenyl-B-D-galactopyranoside (ONPG) (Sigma, St. Louis, MO) or 2·2-azino-di[3-ethyl-benzthiazoline sulfonate] (Kirkegaard & Perry Laboratories, Gaithersburg, MD), was added. Absorbances were read at 410 nm with an MR 5000 ELISA reader (Dynatech, Chantilly, VA). Supernatants from wells containing one single colony were tested repeatedly at different stages of culture.

Inhibition. Before transfer to the appropriately coated ELISA plate, the mAbs were incubated with an equal volume of inhibitor for either 1 or 2 h or overnight at room temperature in a humidified chamber. The inhibitors used were Nε-2,4,-DNP-L-lysine hydrochloride or DNP-ε-aminocaproic acid, and affinity-purified rabbit anti-DNP $F(ab')_2$.

Affinity determination. The determination of affinity constants (K_a) was carried out exactly as described by Friguet.[22] We used the same stock solution of DNP-lysine, the same preparation of affinity-purified rabbit anti-DNP $F(ab')_2$ and the same series of dilutions for all affinity determinations.

Immunoabsorbents. Normal rabbit immunoglobulin, affinity-purified rabbit anti-DNP $F(ab')_2$ and DNP-BSA were immobilized on cyanogen bromide (CNBr)-activated Sepharose 4B (Pharmacia, Piscataway, NY), according to the manufacturer's instructions.

RESULTS

Reactivity of hybrid supernatants and mAbs derived from spleens undergoing a response to DNP-ficoll DNP-Ba or DNP-KLH

Supernatants of cultures obtained by fusion of spleen cells from immune mice and SP2/0 plasmacytoma cells were assayed, simultaneously, for Ab1 and Ab2 reactivity. As a proportion of the positive cultures are sometimes lost upon long-term propagation and cloning, we thought it worthwhile to assess the distribution and the specificity of the antibodies produced in culture early after fusion (and before cloning), as well as after cloning. Therefore, we routinely assayed the supernatants of the fused cultures containing single clones starting 2 to 3 weeks after fusion (referred to as hybrid supernatants).

Two types of antibodies were initially found in the cultures. Some of the hybrid supernatants reacted with DNP-BSA (Ab1) but not with rabbit anti-DNP antibody (Ab2), while others reacted with both DNP-BSA and rabbit anti-DNP antibodies. No supernatants reacting only with rabbit anti-DNP antibodies were found in these trials. The majority of the hybrid supernatants of cultures, derived from the fusion of spleen cells from mice immunized with DNP-Ficoll and SP2/0 plasmacytoma cells, had both Ab1 and Ab2 reactivity, and the proportions of the two types of antibodies remained constant during 5 weeks of culture (Fig. 1a).

The per cent of hybrid supernatants of cultures derived from spleen cells of mice, hyperimmunized with DNP-KLH and showing both reactivities remained in the range of 40 to 60% for up to 7 weeks after fusion (Fig. 1b). Twenty four per cent of hybrid supernatants derived from spleens undergoing a primary response to DNP-KLH had both Ab1 and Ab2 reactivity (Fig. 1c). However, a higher percentage of hybrid supernatants (46%) showed both reactivities when the hybrids were derived from spleens that received a second antigenic stimulation 3 weeks after the primary. The percentage of hybrid supernatants showing both reactivities was higher still (74%) when the hybrids were taken from spleens that received a second antigenic stimulation 11 weeks after the primary. Only 7% of the hybrid supernatants derived from spleens undergoing a primary response to DNP-Ba had both Ab1 and Ab2 reactivity. Because of the limited amounts of supernatant available in 96-well plates, no further characterization of the antibodies could be carried out at this stage. After the cultures were expanded and cloned, however, the monoclonal antibodies (mAbs) could then be characterized for their isotype and their specific reactivity to DNP-BSA and rabbit anti-DNP Abs as well as to a panel of unrelated antigens commonly used to detect polyreactive antibodies (Table 1). Generally, the proportions of mAbs with both Ab1 and Ab2 reactivities were very similar to those obtained from the original hybrid supernatants. Approximately one-half of the mAbs obtained from the primary response to DNP-Ficoll were of the immunoglobulin M (IgM) isotype and were polyreactive. All the mAbs

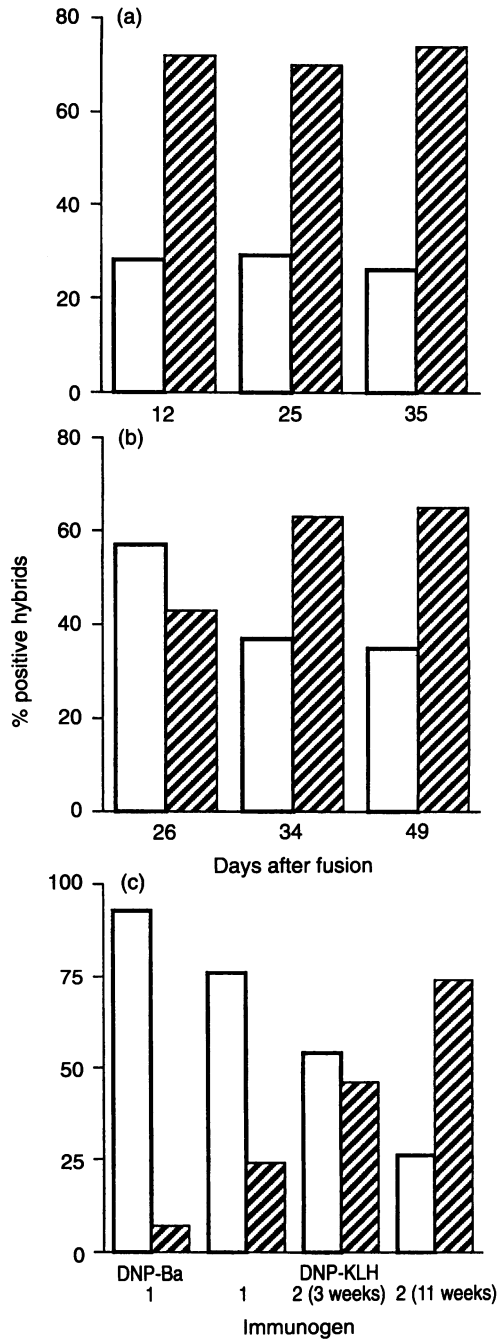

Figure 1. Ab1 (□) and Ab1 and Ab2 (▨) reactivities of hybrid supernatants at different times after fusion of SP2/0 myeloma cells and BALB/c mice spleen cells undergoing a primary response to DNP-Ficoll (a) and a hyperimmune response (to multiple injections) to DNP-KLH (b). Data are from a typical fusion. The reactivity of hybrid supernatants derived from spleen cells of mice undergoing a primary response to DNP-Ba or DNP-KLH (1) or a secondary response to DNP-KLH (2), 5 weeks after fusion, are shown in (c). Data are from 20 fusions. Ab1 reactivity was measured by ELISA on plates coated with DNP-BSA. Ab2 reactivity was measured on plates coated with affinity-purified rabbit anti-DNP $F(ab')_2$. Absorbance readings that were at least three times greater than the average absorbance of medium controls (range 0·00–0·04) or of supernatants obtained from clones of irrelevant specificity (anti-OVA) (range 0·00–0·03) were considered positive. The average absorbances of the supernates from wells containing equivalent number of unfused immunized spleen cells (range 0·00–0·19) was subtracted from the absorbance of supernates from wells containing hybrids (range 0·00–1·47).

Table 1. Specificity and isotype of mAbs derived from the fusion of SP2/0 and spleen cells of BALB/c mice undergoing a primary response to DNP-Ficoll, DNP-Ba and DNP-KLH or a secondary response to DNP-KLH

Antigen and type of immunization	Number (%) of mAbs with: Specificity* Ab1 only	Ab1 + Ab2	Polyreactive	Isotype†
DNP-Ficoll (I°)	25 (52)			IgM 19 (45); IgG1 1 (2); IgG3 2 (5)
		0		
			20 (48)	IgM 20 (48)
DNP-Ba (I°)	25 (81)			IgM 3 (10); IgG2a 7 (22); IgG2b 10 (32); IgG3 3 (10); not typed 2 (6)
		0		
			6 (19)	IgM 6 (19)
DNP-KLH (I°)	37 (75)			IgM 11 (22); IgG1 10 (20); IgG2b 3 (6); IgG3 1 (2); not typed 12 (24)
		6 (12)		IgM 6 (12)
			6 (12)	IgM 4 (8); IgG1 1 (2); IgG2a 1 (2)
DNP-KLH (II°)	11 (19)			IgM 8 (14); IgG1 2 (3); not typed 1 (2)
		41 (71)		IgM 11 (19); IgG1 26 (45); IgG2a 1 (2); IgA 1 (2); IgE 1 (2); not typed 1 (2)
			6 (10)	IgM 6 (10)

*Measured by ELISA on plates coated with DNP-BSA (Ab1) or with affinity–purified rabit anti-DNP $F(ab')_2$ (Ab2).

†Measured by ELISA on plates coated with either DNP-BSA, affinity–purified rabbit anti-DNP $F(ab')_2$ or goat anti-mouse immunoglobulin G (H&L) and developed with a panel of HRPO–labelled goat anti-mouse antibodies with specificity for IgA, IgE, IgM, IgG1, IgG2a, IgG2b and IgG3.

I°, primary response; II°, secondary response.

with double reactivity, derived from spleens undergoing a primary response to DNP-Ba, also reacted with other antigens and thus were polyreactive; in fact they all belonged to the IgM class. Of the 24% of mAbs that had both Ab1 and Ab2 reactivity, and were derived from spleens undergoing a primary DNP-KLH response, half reacted with Ab1 and Ab2, but did not react with other antigens, while the other half were polyreactive. Very few of the mAbs derived from spleens undergoing a secondary response to DNP-KLH were polyreactive (10%); 71% of the mAbs showed exclusively Ab1 and Ab2 reactivity and most of these belonged to immunoglobulin classes other than IgM. These mAb fit the definition of epibodies having strict specificity for both the hapten and for the anti-hapten antibody. The proportions of λ chains for mAbs derived from spleens undergoing a primary response to DNP-Ficoll and secondary responses to DNP-KLH were 52% and 40%, respectively.

Inhibition of epibody binding

Free hapten (DNP-lysine or DNP-caproic acid) is capable of inhibiting both the Ab1 and Ab2 activities of the epibodies (Fig. 2). In the inhibition of Ab1 activity, the hapten competes with the firmly bound hapten–carrier for the combining site of the epibody. In the inhibition of the Ab2 activity, the hapten may bind either the epibodies' combining sites or those of the affinity-purified rabbit anti-DNP coating the wells or both. It was desirable to confirm the involvement of the combining site of the affinity-purified rabbit anti-DNP molecule in the binding of the Ab2 reactivity. Therefore, we investigated whether the Ab2 reactivity was retained when the $F(ab')_2$ fragment of the affinity-purified rabbit anti-DNP was used to coat the wells. Indeed, this was the case, as the $F(ab')_2$ gave activity comparable to that of the whole molecule. No reactivity was found when wells were coated with non-specific rabbit immunoglobulin $F(ab')_2$ or with affinity-purified rabbit anti-OVA $F(ab')_2$, or with affinity purified rabbit anti-chicken immunoglobulin (data not shown). The epibodies also reacted with wells coated with $F(ab')_2$ or Fab fragments of two mouse anti-DNP mAbs possessing exclusively Ab1 reactivity. No reactivity was found when wells were coated with the $F(ab')_2$ fragment of an anti-cocaine mouse mAb (data not shown). In addition, when plates coated with anti-DNP $(Fab')_2$ were incubated with DNP-caproic acid for 2 h, and washed to remove unbound hapten before the addition of the mAbs, the per cent inhibition of binding varied between 55 and 75% (data not shown). Considering the vigorous washing, this is indeed a strong inhibition. We then studied whether the second ligand, rabbit anti-DNP, was also capable of inhibiting both the Ab1 and Ab2 activities of the epibodies. Table 2 shows that both Ab1 and Ab2 activities were completely inhibited by affinity-purified rabbit anti-DNP $F(ab')_2$ as well as by DNP-caproic acid.

One molecule, two reactivities

Even though the monoclonality of the antibodies clearly indicates that both Ab1 and Ab2 activities must be properties of the same molecule, thus defining epibodies, we studied whether the physical removal of the epibody by specific absorption with either insolubilized DNP-BSA or insolubilized affinity-purified rabbit anti-DNP would remove activity for both ligands. The results (Table 3) show that this is indeed the case, confirming that both Ab1 and Ab2 activities are

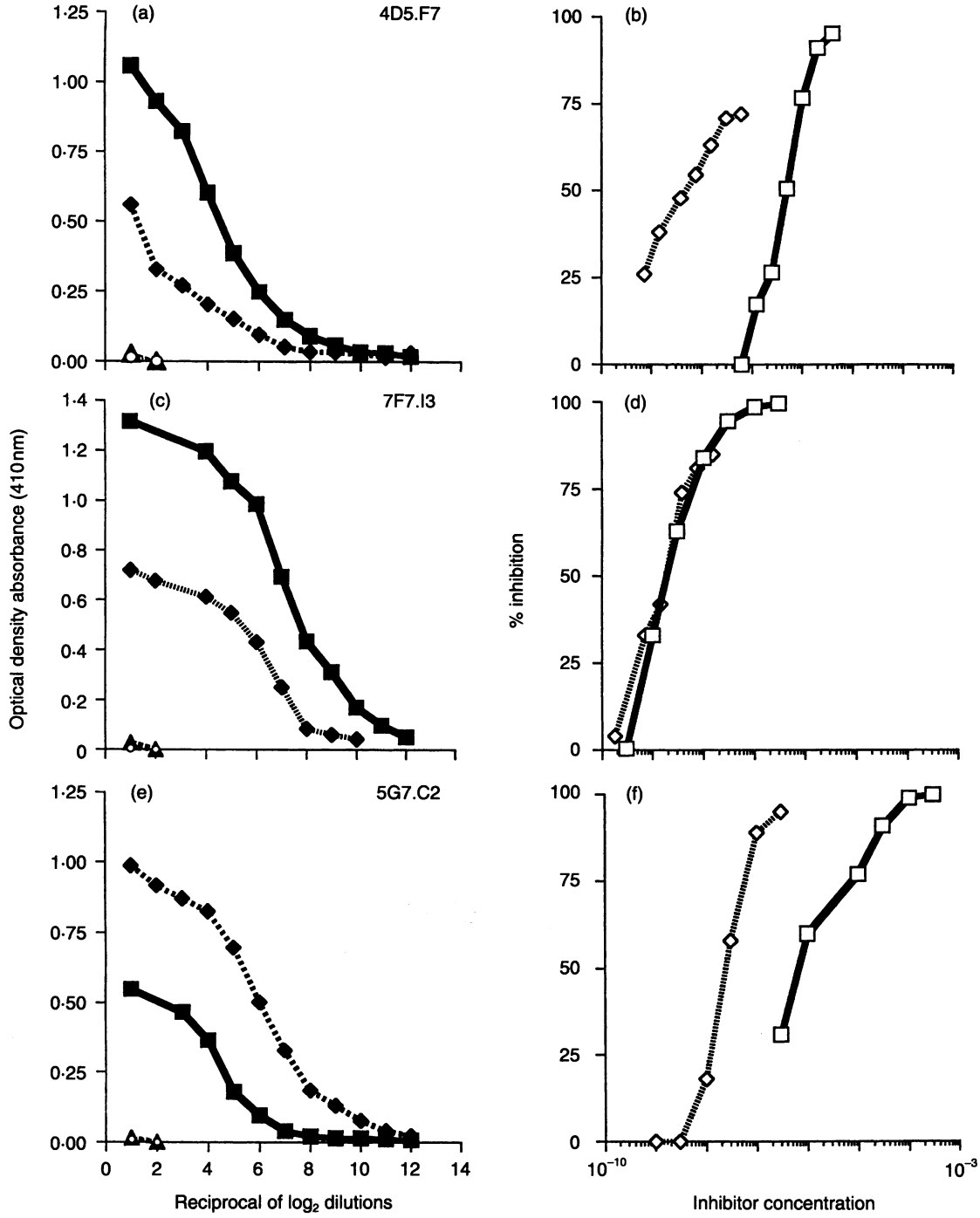

Figure 2. ELISA titration and inhibition curves of mAbs derived from the fusion of DNP-KLH immunized BALB/c spleen cells and SP2/0 myeloma cells. For inhibition, equal volumes of the appropriate concentration of epibody was incubated for 2 hours with serial dilutions of DNP-lysine; 50 µl of the mixture was then transferred to the ELISA plate for testing. (a) 4D5F7 (λγ1) titration started at 3·5 µg/ml. (b) inhibition of Ab1 reactivity was measured at 27 ng/ml, Ab2 reactivity at 350 ng/ml. (c) 7F7·13 (λγ1) titration started at 6·3 µg/ml. (d) inhibition of Ab1 reactivity was measured at 6·3 ng/ml, Ab2 reactivity at 98 ng/ml. (e) 5G7C2 (κµ) titration started at 19·5 µg/ml. (f) inhibition of Ab1 reactivity was measured at 150 ng/ml, Ab2 reactivity at 40 ng/ml. -■-, Ab1 reactivity, titration of supernatants on plates coated with DNP-BSA; -◆-, Ab2 reactivity, titration of supernatants on plates coated with affinity purified rabbit anti-DNP $F(ab')_2$; -▲-, Supernates on wells coated with BSA; -○-, supernates on wells coated with normal rabbit $F(ab')_2$; -□-, per cent inhibition of Ab1 reactivity; -◇- per cent inhibition of Ab2 reactivity.

Table 2. Per cent inhibition of Ab1 and Ab2 activity of selected epibodies by DNP-caproic acid, normal rabbit $F(ab')_2$ or rabbit anti-DNP $F(ab')_2$

	Ab1 (% activity inhibition)*			Ab2 (% activity inhibition)*		
mAb†	DNP-ca	NR $F(ab')_2$	αDNP $F(ab')_2$	DNP-ca	NR $F(ab')_2$	αDNP $F(ab')_2$
5F1.F8	99	0	80	98	31	97
7F7.I3	99	0	96	98	22	96
5B5.H2	98	7	85	97	23	97
4G3.AB7	97	0	49	100	0	70

*The % inhibition of Ab1 and Ab2 activity was determined using DNP-caproic acid (DNP-ca), normal rabbit $F(ab')_2$ (NR $F(ab')_2$) and rabbit anti-DNP $F(ab')_2$ (αDNP $F(ab')_2$) as inhibitors.

†Equal volumes of mAb supernatant and inhibitor were incubated for 60 min at room temperature before transfer to the ELISA plates. The final concentration of inhibitors was $3{\cdot}3 \times 10^{-6}$ M of the $F(ab')_2$, 1×10^{-3} M of the DNP-caproic acid. Second antibody was goat anti-mouse immunoglobulin (H&L) conjugated with horseradish peroxidase. The substrate was ABTS.

Table 3. Elimination of both Ab1 and Ab2 reactivities by absorption of mAb with DNP or anti-DNP immunoabsorbent

	Normal rabbit $F(ab')_2$*		DNP-BSA*		Rabbit anti-DNP $F(ab')_2$*	
mAb†	Ab1 activity	Ab2 activity	Ab1 activity	Ab2 activity	Ab1 activity	Ab2 activity
7F7.I3	87	71	10	9	0	21
5G7.C2	99	100	0	10	0	0
5B5.H2	98	100	3	2	0	21
4D3.G7	100	100	9	0	0	0

*Figures refer to the % unabsorbed control remaining following absorption with normal rabbit $F(ab')_2$, DNP-BSA or rabbit anti-DNP $F(ab')_2$.

†mAbs were batch absorbed twice with immunoabsorbents made with normal rabbit $F(ab')_2$ or affinity-purified rabbit anti-DNP $F(ab')_2$ or DNP-BSA conjugated with cyanogen bromide-activated Sepharose 4B, then tested on ELISA plates coated with DNP-BSA (Ab1 activity) or with affinity-purified anti-DNP rabbit $F(ab')_2$ (Ab2 activity).

properties of the same epibody molecule. Insolubilized normal rabbit $F(ab')_2$ did not remove any activity.

Determination of epibody affinity

It was of interest to determine and compare the affinity constants of the epibodies for the two specific ligands: DNP-lysine as the ligand for the Ab1 function and affinity-purified rabbit anti-DNP for the Ab2 function. The association constants for the Ab1 and Ab2 functions were derived from Klotz plots constructed from the data obtained for the fraction of bound antibody as a function of the total antigen concentration. The association constants for the Ab1 and Ab2 functions for several epibodies are listed in Table 4. Contrary to expectations, in most cases affinity constants for the Ab2 activity were higher than those for Ab1 activity.

Self-reactivity of epibodies

In order to establish if these epibodies would bind themselves, two epibodies were purified on an anti-mouse immunoglobulin Sepharose column and biotinylated. As shown in Table 5, they both bind themselves and each other, while they do not bind mAbs with irrelevant specificities but corresponding isotypes.

Table 4. Ab1 and Ab2 K_a for a panel of mAbs with Ab1 and Ab2 activity derived from mice immunized with DNP-KLH

		K_a	
mAb	Isotype	Ab1	Ab2
4A3.M2	κγ1	$4{\cdot}84 \times 10^4$	$3{\cdot}85 \times 10^7$
4D3.G7	λγ1	$2{\cdot}61 \times 10^7$	$2{\cdot}97 \times 10^8$
5F1.F8	κγ1	$1{\cdot}7 \times 10^7$	$2{\cdot}34 \times 10^8$
4D5.F7	λγ1	$1{\cdot}25 \times 10^7$	$2{\cdot}7 \times 10^8$
4G3.AB7	λμ	$5{\cdot}1 \times 10^7$	$1{\cdot}09 \times 10^8$
5H3.G3	λγ1	$2{\cdot}5 \times 10^6$	$1{\cdot}37 \times 10^7$
7F7.I3	λγ1	$1{\cdot}96 \times 10^8$	$6{\cdot}4 \times 10^8$
5H2.B4	κγ1	5×10^7	$3{\cdot}14 \times 10^8$
7C7.D2	κγ1	$7{\cdot}97 \times 10^6$	$1{\cdot}8 \times 10^7$
8E1.H11	κγ1	$2{\cdot}1 \times 10^6$	$4{\cdot}6 \times 10^6$
5B5.H2	λγ1	4×10^8	$2{\cdot}3 \times 10^8$

Selected mAbs were incubated with serial dilutions of inhibiting hapten (for Ab1 reactivity) and rabbit anti-DNP $F(ab')_2$ (for Ab2 reactivity) until equilibrium. The free antibody was measured by ELISA and using Klotz plots, and the association constant K_a for each mAb was determined using the method of Friguet.[32]

DISCUSSION

In order to better understand the unexpected observation that Ab1 and Ab2 responses to DNP are similar with respect to both kinetics and magnitude,[1] we studied two main features of the response to DNP. First, we investigated the percentage of the elicited antibodies that were endowed with Ab1 reactivity only (i.e. were monospecific) and the percentage that had both Ab1 and Ab2 reactivity (i.e. were epibodies or polyreactive). This aspect of the response was best examined soon after

Table 5. Reactivity of purified epibodies on different coatings*

mAb	Isotype	Absorbance on ELISA plates coated with: DNP-BSA	αDNP F(ab')$_2$	4D3G7	5G7C2	RPC (IgG)	TEPC (IgM)
4D3.G7	IgG	1·718	0·561	0·367	0·346	0·017	0
5G7.C2	IgM	0·679	0·220	0·218	0·300	0·028	0·017

*ELISA plates were coated with DNP-BSA, anti-DNP F(ab')$_2$, 4D3G7, 5G7C2, RPC (IgG) or TEPC (IgG), and incubated with biotinylated epibodies used at an approximate concentration of 1×10^{-9} M. The assay was developed with avidin conjugated horseradish peroxidase. The substrate was ABTS. RPC and TEPC, mAbs of irrelevant specificity but corresponding isotypes, were used as control coatings.

fusion of the immune cells with plasmacytoma cells, before the inevitable losses of positive cultures, which occur during expansion and cloning, distort the picture. Strictly speaking, therefore, the percentage of antibodies in hybridoma supernatants with both Ab1 and Ab2 reactivity could reflect the presence of more than one clone in the wells tested. However, the probability of two productive clones being present in the same well is low to start with, as the majority of cultures contained clones that tested negative for Ab1 and/or Ab1 and Ab2 activity. In addition, we were careful to ascertain, by microscopic examination, that all cultures tested contained only a single developing clone. A second possible source of error, inherent in this early testing strategy, is that some of the antibodies in the culture supernates were the product of unfused immune cells, rather than of hybridoma cells. To control for this possibility, the average absorbance reading of wells containing a comparable number of unfused spleen cells was subtracted from the reading obtained from wells containing hybridoma cells.

Second, we investigated the nature and specificity of the antibodies produced in response to DNP linked to T-independent and T-dependent carriers, and the effects of the intensity and length of immunization on the characteristics of the antibodies. This required the production of relatively large amounts of mAbs. Clearly, the similarity of the kinetics and magnitude of the Ab1 and Ab2 responses that we reported earlier, especially for mice immunized with DNP-Ficoll,[1] can be explained by the production of molecules with both Ab1 and Ab2 reactivity, as well as polyreactivity to a number of unrelated antigens of the IgM isotype.

The nature of the carrier plays a role in the type and specificity of the antibody produced. In contrast with the results obtained with the T-independent type 2 carrier Ficoll, immunization with the T-independent type 1 carrier *B. abortus* resulted in antibodies, most of which were strictly specific for the nominal antigen only. The isotype distribution of these antibodies included IgM and three subtypes of IgG. Polyreactive antibodies were also represented, albeit in a minor way, and were of the IgM isotype, as in the case of DNP-Ficoll.

The role of length of immunization in the changing pattern of the antibody response can best be discerned in the response to DNP-KLH. The response to DNP-KLH clearly shows a time-dependent shift in the specificity and isotype of the antibody produced, with Ab1 reactivity only predominant in the primary response and with epibodies capable of binding both the nominal antigen and its specific antibody becoming prevalent in later stages of the immune response. In view of this unexpected finding it was desirable to characterize these epibodies and ensure that a single epibody molecule was capable of binding both ligands and that the idiotope bound by the epibody was located in the combining site of the idiotypic antibody. That both anti-hapten (Ab1) and anti-anti-hapten (Ab2) reactivities were functions of the same molecule was indicated by the monoclonal nature of the epibodies and by the ability of insolubilized DNP-BSA or insolubilized anti-DNP antibody to remove reactivity for both ligands. That the Ab2 reactivity of the epibody involves the antigen-combining site of the epibody itself, as well as that of the idiotypic rabbit anti-DNP antibody, was indicated by:

1 the ability of the hapten to inhibit the reaction, not only when the mAb was preincubated with the hapten, but also when the coated plate was preincubated with the hapten, and unbound hapten was removed by washing; and

2 the observation that epibodies bind not only to the whole molecule of rabbit anti-DNP antibody, but also to its F(ab')$_2$ fragment as well as to the F(ab')$_2$ or the Fab fragments of mouse anti-DNP antibodies (but not those derived from rabbit or mouse antibodies with other specificities).

As the only shared feature of the rabbit and mouse F(ab')$_2$s that react with the epibodies is the specificity for DNP, these results confirm the involvement of the antigen-combining sites in the Ab2 reactivity. Xenogeneic Ab1 is a commonly used reagent for detecting Ab2.[23] Its use limits the system to the detection of Ab2β, those Ab2s possessing the internal image of the antigen.[24] As mouse and rabbit anti-DNP antibodies (Ab1) share a common antigenic specificity, they are likely to have similar antigen-combining sites or paratopes; however, they are not likely to share interspecies cross-reactive idiotypes, which have been detected only rarely and are not associated with a given specificity.[25] The high proportion of mAbs with λ chain is not surprising in this particular case; similar results were obtained when the isotypes of antibodies produced by single spleen cells undergoing anti-DNP responses were assessed by ELISA spot.[1]

The panel of epibodies used for affinity determination had a higher affinity for rabbit anti-DNP than for DNP. Affinity constants were determined by the method of Friguet,[22] which has been validated for a number of ligands, including immunoglobulin molecules.[26,27] The 11 epibodies chosen for affinity determination are by no means a representative sample of the many cells undergoing the response. The technique requires that the concentration of Ab to be tested should be kept as low as possible, but still be detectable by the assay. This requirement might favour the selection of higher affinity Ab2.

In most cases reported, the idiotope with which the epibody reacts is separate and distinct from the combining site of the idiotope-bearing antibody. In fact, the only report that we

found in the literature of an epibody with the characteristics we have described has been described by Kang & Kohler,[28,29] who raised an anti-T15 mAb which reacted specifically with both T15 (an anti-phosphoryl choline myeloma protein) and with phosphoryl choline, the nominal hapten. Because of its potential for self-binding, this special type of epibody was termed autobody.[28,29] The possible presence of epibodies with specificity for the antigen as well as for a configuration complementary to the antigen, in a conventional immune response, is suggested by the findings of Forsyth & Hoffmann.[9] They found that hyperimmune chicken anti-BSA sera bound not only BSA (the antigen) but also affinity-purified anti-BSA antibodies obtained from sera of hyperimmunized chickens; in addition, these hyperimmune sera bound affinity-purified anti-BSA antibodies of mouse origin, but not affinity purified anti-KLH or anti-DT (diphtheria toxoid) chicken antibodies. The idiotypic activity co-purified with the anti-idiotypic activity on a BSA-sepharose column. They calculated that the concentration of idiotypic binding antibodies in hyperimmune sera was about 50 to 100% of that of the anti-BSA antibodies. The percentage was much lower in sera after primary immunization. From inhibition experiments they also concluded that all hyperimmune sera had a greater intrinsic affinity for the binding of the anti-idiotypic antibody than for BSA. It is very likely that their results are indeed caused by the presence of double-reactive antibodies, similar to the ones we have described in this paper, rather than polyreactive antibodies. In fact, the reactivities they describe separate with molecules of the size of IgG, and also show relatively low binding reactivity with KLH and DT.

We know very little of the configuration by which the epibody achieves its double reactivity, or of the significance of a high proportion of double-reactive antibodies in the mature immune response. Molecules such as these have a potential for self-regulation. Antibodies similar to those described in this study may contribute very little to the concept of a wide-ranging, high connectivity network that constitutes the immune system, as visualized by Jerne.[30] However, such molecules may contribute to the regulation of the network, by allowing the idiotypic cascade to cycle back on itself. Dwyer,[31] in a different context, has proposed that some epibodies can be considered a subset of super organizer antibodies. He described 'super organizer' antibodies as 'the shock absorbers of the immune system because they dampen the reverberations in idiotypic networks set in motion by the introduction of the antigens'. Although the antibodies we describe here have characteristics different from Dwyer's superorganizers, they could represent a component of a more limited and specific regulatory system. On the other hand, clones that produce antibodies which react with the antigen and with the combining site of an antibody specific for the nominal antigen have the advantage of being stimulated by both the antigen and the antibody to the antigen and therefore their preferential stimulation may be prolonged, even when the antigen is no longer present. The fact that we see an increased proportion of these epibodies later in the secondary response supports this hypothesis.

It is interesting to speculate on the selective pressures that may drive these changes in the pattern of the immune response. In the primary response, regardless of the nature of the carrier, antibodies with Ab1 activity only are well represented, as are polyreactive antibodies. At this time the antigen is probably present in large enough concentrations so that selection among clones of responding cells does not play a major role. All cells with receptors capable of binding the antigen have an equal chance of being stimulated. In later stages of the immune response, however, not only is the antigen reintroduced into the environment of the responding cells with the secondary immunization, but the specific antibody produced during the primary response is also present. Clones of cells with receptors capable of binding both the nominal antigen and its specific antibody would be activated at this time, resulting in the production of epibodies. Antibodies with strict specificity for the nominal antigen, as well as polyreactive antibodies, are still being produced, but constitute a smaller portion of the immune response. In any case it is likely that epibodies with Ab1 and Ab2 reactivities are normal components of conventional immune responses.

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REFERENCES

1. Segre M. & Segre D. (1994) Behavior of the idiotypic network in conventional immune responses. III. Detection and enumeration of cells producing idiotypic and anti-idiotypic antibodies by a spot ELISA technique. *Cell Immunol* **159,** 40.
2. Bona C.A., Finley S., Waters S. & Kunkel H.G. (1982) Anti-immunoglobulin antibodies. III. Properties of sequential anti-idiotypic antibodies to heterologous anti-γ globulins. Detection of reactivity of anti-idiotype antibodies with epitopes of Fc fragments (homobodies) and with epitopes and idiotopes (epibodies). *J Exp Med* **156,** 986.
3. Bona C.A., Kang C-Y., Kohler H. & Monestier M. (1986) Epibody: the image of the network created by a single antibody. *Immunol Rev* **90,** 115.
4. Chen P.P., Fong S., Houghten R.A. & Carson D.A. (1985) Characterization of an epibody. An anti-idiotype that reacts with both the idiotype of the rheumatoid factors (RF) and the antigen recognized by RF. *J Exp Med* **161,** 323.
5. Hunt Gerardo S., Persselin J.E. & Stevens R.H. (1990) Human IgG anti-F(ab')$_2$ antibodies possess rheumatoid factor activity. *Clin Exp Immunol* **81,** 293.
6. Fischel R. & Eilat D. (1992) Structure and binding properties of a monoclonal anti-idiotypic, auto-antibody to anti-DNA with epibody activity. *J Immunol* **149,** 3089.
7. Puccetti A., Migliorini P., Sabbaga J. & Madaio M.P. (1990) Human and murine anti-DNA antibodies induce the production of anti-idiotypic antibodies with autoantigen-binding properties (epibodies) through immune-network interactions. *J Immunol* **145,** 4229.
8. Dwyer D.S., Vakil M. & Kearney J.F. (1986) Idiotypic network connectivity and a possible cause of myasthenia gravis. *J Exp Med* **164,** 1310.
9. Forsyth R.B. & Hoffmann G.W. (1990) A study of auto-anti-idiotypes to BSA. *J Immunol* **145,** 215.
10. Pateraki E., Kaklamani E., Kaklamanis P., Portocalas R. & Aessopos A. (1986) Autoantibodies in systemic lupus erythematosus and normal subjects. *Clin Rheumat* **5,** 338.
11. Ishigatsubo Y., Sakamoto H., Hagiwara E. *et al.* (1989)

Quantitation of auto-antibody-secreting B cells in systemic lupus erythematosus. *Autoimmunity* **5,** 17.

12. Mayer R., Zaghouani H., Usuba O. & Bona C. (1990) The Ly-1 gene expression in murine hybridomas producing autoantibodies. *Autoimmunity* **6,** 293.
13. Kaushik A., Lim A., Poncet P., Ge X.R. & Dighiero G. (1988) Comparative analysis of natural antibody specificities among hybridomas originating from spleen cell and peritoneal cavity of adult NZB and BALB/c mice. *Scand J Immunol* **27,** 461.
14. Glotz D., Sollazzo M., Riley S. & Zanetti M. (1988) Isotype, VH genes, and antigen-binding analysis of hybridomas from newborn normal BALB/c mice. *J Immunol* **141,** 383.
15. Segre M. & Segre D. (1976) Humoral immunity in aged mice. I. Age-related decline in the secondary response to DNP of spleen cells propagated in diffusion chambers. *J Immunol* **116,** 731.
16. Rittenberg M.B. & Pratt K.L. (1969) Antitrinitrophenyl (TNP) plaque assay. Primary response of BALB/c mice to soluble and particulate immunogen. *Proc Soc Exp Biol Med* **132,** 575.
17. Schlueter A.J., Segre D., Kuhlenschmidt M.S. & Segre M. (1992) Behavior of the idiotypic network in conventional immune responses. I. Kinetics of idiotypic and anti-idiotypic antibodies following immunization with T-dependent antigens. *Cell Immunol* **144,** 311.
18. Oi V.T. & Herzenberg L.A. (1980) Immunoglobulin-producing hybrid cell lines. In: *Selected Methods in Cellular Immunology* (eds B. B. Mishell & S. M. Shiigi), p. 354. W. H. Freeman and Company, San Francisco, CA.
19. Reik L.M., Maines S.L., Ryan D.E., Levin W., Bandiera S. & Thomas P.E. (1987) A simple non-chromatographic purification procedure for monoclonal antibodies. Isolation of monoclonal antibodies against cytochrome P450 isozymes. *J Immunol Methods* **100,** 123.
20. Andrew S.M. & Titus J.A. (1997) Fragmentation of immunoglobulin G. In: *Current Protocols in Immunology* (eds J. E. Coligan, A. M. Kruisbeek, D. H. Marguiles, E. M. Shevach & W. Strober), Vol. 1, p. 2.8.1. John Wiley & Sons, Inc., New York.
21. Stocker J.W., Malavasi F. & Trucco M. (1981) Enzyme immunoassay for the detection of hybridoma products. *Immunological Methods* (eds I. Lefkovits & B. Pernis), Vol. 2, p. 303. Academic Press, New York.
22. Friguet B., Chafotte A.F., Djavadi-Ohaniance L. & Goldberg M.E. (1985) Measurements of true affinity constant in solution of antigen-antibody complexes by enzyme-linked immunosorbent assay. *J Immunol Methods* **77,** 305.
23. Reilly T.M. & Root R.T. (1986) Production of idiotypic and anti-idiotypic antibodies by BALB/c mice in response to immunizations with glucagon, vasopressin or insulin: Supporting evidence for the network concept. *J Immunol* **137,** 597.
24. Nisonoff A. & Lamoyi E. (1981) Implication of the presence of an internal image of the antigen in anti-idiotypic antibodies: Possible implication to vaccine production. *Clin Immunol Immunopathol* **21,** 397.
25. Bona C.A. (1981) *Idiotypes and Lymphocytes.* p. 12. Academic Press, New York.
26. Van Der Heijden R.W.J., Bunschoten H., Hoek A. *et al.* (1991) A human $CD5^+$ B cell clone that secretes an idiotype-specific high affinity IgM monoclonal antibody. *J Immunol* **146,** 1503.
27. Monestier M., Bonin B., Migliorini P. *et al.* (1987) Autoantibodies of various specificities encoded by genes from the V_H J558 family bind to foreign antigens and share idiotopes of antibodies specific for self and foreign antigens. *J Exp Med* **166,** 1109.
28. Kang C.Y. & Kohler H. (1986) Immunoglobulin with complementary paratope and idiotope. *J Exp Med* **163,** 787.
29. Kang C.Y., Cheng H.L., Rudikoff S. & Kohler H. (1987) Idiotypic self binding of a dominant germline idiotype (T 15). Autobody activity is affected by antibody valency. *J Exp Med* **165,** 1332.
30. Jerne N.K. (1984) Idiotypic networks: and other preconceived ideas. *Immunol Rev* **79,** 5.
31. Dwyer D.S. (1988) Idiotype connectivity of antibody response specific for self and nonself antigens. In: *Biological Applications of Anti-idiotypes* (ed. C. A. Bona), Vol. 2, p. 55. CRC Press, NW Boca Raton, FL.