Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 1194–1198, February 1985
Immunology

NZB mouse system for production of monoclonal antibodies to weak bacterial antigens: Isolation of an IgG antibody to the polysaccharide capsules of *Escherichia coli* K1 and group B meningococci

(bacterial capsules/*N*-acetylneuraminic acid/ELISA)

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Communicated by I. C. Gunsalus, September 27, 1984

ABSTRACT **A system for the production of monoclonal antibodies, particularly of the IgG type, against weakly immunogenic bacterial polysaccharide antigens is described. This system, which is based on the autoimmune NZB mouse strain, has been used to produce a monoclonal IgG2a antibody against the meningococcus group B and *Escherichia coli* K1 polysaccharides, identical homopolymers of α(2→8)-linked units of *N*-acetylneuraminic acid that are extremely poor immunogens. Comparison of the humoral immune responses of normal BALB/c mice and autoimmune NZB mice to hyperimmunization with group A, B, and C meningococci showed that, although both strains mounted a weak meningococcal B polysaccharide-specific IgM response, only the NZB strain mounted an IgG response. Similarily, NZB mice mounted a stronger IgG response to the more immunogenic group C meningococcal polysaccharide than did BALB/c mice, although this difference was less pronounced than that observed with meningococcal B polysaccharide. No difference between the two strains of mice was demonstrable with the strongly antigenic group A meningococcal polysaccharide. These results indicate that the NZB system may be generally useful for the production of monoclonal antibodies against weakly antigenic bacterial determinants.**

A serious problem in individuals suffering from certain infectious diseases, especially those produced by some invasive Gram-negative bacteria, is that a strong humoral immune response against major cell-surface structures of the infectious agents is not achieved. Some types of poorly immunogenic bacterial capsules are almost certainly responsible for this weak response, either because they interfere with cooperative cellular events necessary for induction of an antibody response (1) or because their chemical structures resemble those of host determinants (antigenic mimicry) and thus are not recognized as foreign (2). Poor immunogenicity results in escape of the pathogen from antibody-mediated host defenses, including certain complement-dependent bactericidal mechanisms and phagocytic events.

Among invasive Gram-negative bacteria, *Escherichia coli* K1 and meningococcus group B strains, the capsules of which are chemically and immunologically identical (3, 4), produce serious and often fatal infections: about 80% of the cases of neonatal *E. coli* meningitis and 36% of those of neonatal sepsis are caused by $K1^+$ strains of *E. coli* (5), whereas >80% of cases of meningococcal meningitis in European countries are caused by *Neisseria meningitidis* group B strains (6). Individuals infected with these bacteria have difficulty in producing antibodies against their very poorly immunogenic capsules (7, 8). If antibodies to the capsules are produced at all, they are always of the IgM isotype. In fact, to our knowledge, only one high-titer antiserum against meningococcal group B polysaccharide (MBPS), induced in a horse by J. B. Robbins (described in ref. 9), has been raised experimentally in an animal. Several attempts have been made to increase the immunogenicity of capsular polysaccharides by coupling them to protein carriers (8, 10–13). These experiments demonstrated that active immunization with K1 polysaccharide-protein conjugates (14) and passive protection with antibodies (15, 16) improved the survival of experimental animals challenged with *E. coli* K1 strains. However, no successful vaccination scheme has been established for MBPS. Although the virulence of invasive *E. coli* K1 and meningococcus B strains is undoubtedly multifactorial, the capsule clearly plays a pivotal role.

One important experimental tool for the investigation of molecular mechanisms of biological activities is monoclonal antibodies. However, the production of monoclonal antibodies against weakly immunogenic structures such as the K1 capsule and MBPS, which are identical homopolymers of *N*-acetylneuraminic acid (Neu5Ac), has been extremely difficult and, for IgG-type antibodies, so far unsuccessful. We now have circumvented this difficulty by employing NZB mice, which exhibit immunological hyperreactivity, and describe here the generation of a high-titer IgG2a monoclonal antibody that is specific for the K1 and meningococcus B capsules.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. *E. coli* D1737 K1, D703 (a K^- mutant of D1737 K1), 016:K1, and C375 (0132:K1) were gifts from F. Ørskov (Copenhagen) (9), whereas 04:K12, 06:K13, and 06:K14 were gifts from K. Jann (Freiburg, F.R.G.). 01:K1 and 073:K92 are urinary tract infection isolates from the diagnostic laboratory of the Institute of Medical Microbiology, University of Mainz. $01:K^-$; $016:K^-$, and $132:K^-$ were isolated from the corresponding K1 strains as mutants resistant to K1-specific bacteriophage (phages kindly provided by B. Rowe, London; see ref. 17). LE392 is a derivative of the laboratory strain *E. coli* K-12. Group A, B, and C meningococci were obtained from the American Type Culture Collection (nos. 13077, 13090, and 13102, respectively). Plasmid pKT274 is a hybrid cosmid carrying the cloned genes for production of K1 capsule (6), whereas pGB33 and

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Abbreviations: MBPS, meningococcal group B polysaccharide; Neu5Ac, *N*-acetylneuraminic acid; KLH, keyhole limpet hemocyanin.

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pGB44 are gamma-delta insertion mutant derivatives of pKT274 (unpublished results).

Bacterial Capsular Polysaccharides. Highly purified meningococcal A, B, and C capsular polysaccharides were obtained from Connaught Laboratories (Swiftwater, PA). *E. coli* K1 polysaccharide was kindly provided by K. Jann (Freiburg).

Immunization Scheme for Comparison of the Immune Responses of NZB and BALB/c Mice. Four- to five-week-old NZB and BALB/cAnN mice (Zentralinstitut für Versuchstierkunde, Hannover, F.R.G.) of both sexes were injected intraperitoneally with 10^8 live group A, B, or C meningococci in complete Freund's adjuvant. Booster injections were given on days 7 and 13, again with 10^8 meningococci, and on days 19, 25, and 31, with 2×10^8 meningococci. Animals were bled from the retroorbital plexus on day 5 after primary immunization and 3 days after the last booster injection. The sera were collected and stored at −70°C until used.

Production of Monoclonal Antibodies. Three-week-old female NZB mice (The Jackson Laboratory) were immunized intraperitoneally twice a week over a period of 4 weeks with 5×10^8 live group B meningococci (ATCC 13090). The primary injection was given with complete Freund's adjuvant (Difco). At the end of the immunization regime, the mice were splenectomized, and the spleen cells were fused with X63-Ag8.653 myeloma cells (18) as described (19). The resulting hybridomas were cloned by limiting dilution. For production of ascites fluids, hybridoma cells were injected intraperitoneally into Pristane-pretreated BALB/cAnN mice, irradiated with 300 rad (3 Gy).

ELISA for Detecting Monoclonal Antibodies Against Group B Meningococcal Antigen. MBPS was covalently coupled to keyhole limpet hemocyanin (KLH) (Calbiochem) by the method of Schneerson *et al.* (12). Microtiter plates (Costar "V" vinyl plates) were incubated with 20 μl of KLH-MBPS (final polysaccharide concentration, 100 μg/ml) for 2.5 hr at room temperature. The microtiter plates were washed twice with 200 μl of 10 mM K_2HPO_4/KH_2PO_4, pH 7.5/15 mM NaCl (buffer A) and then incubated with buffer A containing 2% (wt/vol) gelatin for 20 min. Hybridoma culture supernatant fluids (20 μl) then were added, and plates were incubated for 1 hr at room temperature and then washed extensively prior to addition of 10 μl of peroxidase-labeled anti-mouse immunoglobulin (Dako, Munich, diluted 1:500) and further incubation for 1 hr. The microtiter plates then were washed twice with buffer A containing 2% gelatin and once with 10 mM potassium phosphate buffer, pH 6.0. Substrate solution (20 μl of 2.7% 2,2′-azino[di(3-ethylbenzthiazolinesulphonic acid)] [Sigma] in potassium phosphate, pH 6, containing 0.0025% H_2O_2) then was added to each well, and the peroxidase reaction was measured by the increase in absorbance at 414 nm, using a Titertek Multiskan MC (Flow Laboratories, Meckenheim, F.R.G.).

ELISA Tests for Characterization of the Monoclonal Antibody. Microtiter plates were precoated with poly(D-lysine) (25 μg/ml; Sigma) in buffer A for 1 hr at room temperature. After the plates had been washed three times with buffer A, 20 μl of a suspension of bacteria (10^8 per ml) that had been grown for 4 hr in tryptic soy broth or of purified meningococcal group A, B, or C or *E. coli* K1 polysaccharides (20 μg/ml in buffer A) was added and plates were incubated for 1 hr at room temperature. Bacteria then were fixed by addition of glutaraldehyde (0.05%). Plates were washed three times with buffer A and once with 2% gelatin in buffer A, after which the same procedure described above for KLH-MBPS was followed.

ELISA for Testing BALB/c and NZB Mouse Sera. Microtiter plates were coated with purified capsular polysaccharides of meningococci A, B, or C as described above. To differentiate IgM- and IgG-class antibodies, sera were made 0.1 M in 2-mercaptoethanol. Sera then were diluted in 2% gelatin solution and tested in ELISA as described above.

Precipitation and Agglutination. Immunoelectrophoresis and immunodiffusion were done in 1% agarose. K1 antigen for immunoelectrophoresis was isolated as follows: *E. coli* D1737 K1 bacteria were cultured overnight at 37°C in 300 ml of tryptic soy broth supplemented with 75 mg of glucose. The culture was centrifuged at $3000 \times g$, and the supernatant fluid was incubated with 600 mg of hexadecyltrimethylammonium bromide (Sigma) (20) for 8 hr at 4°C before centrifugation at $6000 \times g$. The resulting pellet was dissolved in 10 ml of 1 M $CaCl_2$ and extensively dialyzed and concentrated against 1 M $CaCl_2$, after which it was dialyzed against buffer A. Halo precipitin formation with horse antiserum to *N. meningitidis* B (generously provided by J. Robbins, Bethesda, MD) and with monoclonal antibody 735D4 was done as described (9). Agglutination was carried out with group B meningococci or *E. coli* 0132:K1 bacteria and ascites fluid of clone 735D4.

RESULTS

Use of NZB Mice to Induce an Anti-K1/MBPS Monoclonal IgG. Before the generation of the monoclonal antibody described below, several unsuccessful attempts to obtain an anti-K1 monoclonal antibody had been carried out and more than 3000 culture supernatant fluids had been tested. These attempts had involved the hyperimmunization of BALB/cAnN mice with K1 polysaccharide in different forms: formalin-fixed *E. coli* 0132:K1 and 01:K1 bacteria, K1-antigen (colominic acid, Sigma) covalently coupled to KLH or bovine serum albumin (10, 12), and immunoprecipitates of MBPS obtained with horse anti-meningococcus B serum.

To test another approach, NZB mice were used with the idea that these autoimmune animals, which exhibit hyperactivity of the B- and T-cell systems, might give a positive response to weak antigens. Although a first fusion with spleen cells of an NZB mouse immunized with formalin-treated *E. coli* D1737:K1 bacteria failed to produce any K1-positive hybridoma clones, a second fusion with spleen cells of an NZB mouse immunized with viable group B meningococci (see *Materials and Methods*) was successful. Of 303 culture supernatant fluids from this fusion that were tested, one (designated 735D4) gave a positive reaction with meningococcal B polysaccharide. By use of class- and subclass-specific, peroxidase-conjugated anti-mouse immunoglobulins (Nordic, Tilburg, The Netherlands) in the KLH-MBPS ELISA, the heavy chain of the monoclonal antibody was shown to be a γ_{2a}, whereas its light chain was determined to be κ. The monoclonal antibody was shown to strongly bind peroxidase-labeled protein A (Sigma).

The specificity of monoclonal antibody 735D4 for the K1 polysaccharide and MBPS was verified by examination of its reaction spectrum in a bacterial ELISA. As shown in Table 1, the antibody reacted positively with all *E. coli* K1 and group B meningococcal strains tested but negatively with K^-

Table 1. Reactivity of monoclonal antibody 735D4 in ELISA with various bacteria and bacterial polysaccharides

	Positive	Negative	
E. coli	D1737:K1	D703 (K^-)	04:K12
	01:K1	01:K^-	06:K13
	016:K1	016:K^-	06:K14
	0132:K1	0132:K^-	073:K92
Meningococci	B	A, C	
Polysaccharides	KLH-MBPS *E. coli* K1		

mutant derivatives of the $K1^+$ isolates; *E. coli* strains having capsular types K12, K13, K14, and K92; and group A and C meningococci. The capsular polysaccharides of group C meningococci and of *E. coli* K92 strains are polymers of Neu5Ac in $\alpha(2\rightarrow9)$ linkage and in alternating $\alpha(2\rightarrow8)$ and $\alpha(2\rightarrow9)$ linkage, respectively (21). We therefore conclude that an as yet undefined number of repeating $\alpha(2\rightarrow8)$-linked Neu5Ac units represents the antigenic determinant for our monoclonal antibody.

The Anti-K1/MBPS Monoclonal Antibody Does Not Distinguish *O*-Acetylated from Non-*O*-Acetylated Capsular Material. Strains C375, 01:K1, and D1737 all produce a K1 polysaccharide capsule, the Neu5Ac units of which are *O*-acetylated (OAc^+) to various extents, whereas strains 016:K1 and meningococcus B have capsules that are not *O*-acetylated (OAc^-) (9). In the ELISA of strains C375 (0132:K1) and 016:K1 and their corresponding K^- mutant derivatives, no significant difference between the two $K1^+$ strains was detected (Fig. 1), which suggests that the monoclonal antibody does not distinguish *O*-acetylated polysaccharide from the non-*O*-acetylated form. This result is similar to that of Ørskov *et al.* (9), who found antibodies against both OAc^+ and OAC^- K1 strains after immunization of rabbits with an OAc^- strain, whereas they found antibodies mostly against *O*-acetylated polysaccharide in rabbits immunized with OAc^+ strains.

Reaction of the Anti-K1/MBPS Monoclonal Antibody with Isogeneic $K1^+/K1^-$ Bacteria. Plasmid pKT274 is a cosmid derivative carrying genes for the production of K1 capsular material (6). Plasmids pGB33 and pGB44 are gamma-delta insertion mutant derivatives of pKT274 that specify the synthesis of K1 polysaccharide but not the transport of this material to the cell surface; bacteria carrying these mutant plasmids accumulate K1 polysaccharide intracellularly (unpublished observations). Fig. 2*a* shows the results of ELISA of the monoclonal antibody with *E. coli* K-12 strain LE392 bacteria and with derivatives of this strain carrying pKT274, pGB33, or pGB44. Bacteria carrying pKT274 were positive, whereas bacteria lacking the plasmid were negative. Bacteria carrying pGB33 or pGB44 showed only small positive reactions, although, after cell lysis by sonication, K1 antigen production by the former was found to be similar in extent to that of bacteria carrying pKT274 (Fig. 2*b*). The antibody reaction indicated lower amounts of K1 antigen in lysed bacteria carrying pGB44, and, in this instance, a pronounced prozone phenomenon was observed. However, modification of the ELISA, so that the monoclonal antibody was used to coat the solid phase, sonicated pGB44-carrying bacteria constituted the liquid phase, and bound antigen was detected with β-galactosidase-labeled monoclonal anti-K1 antibody, led to disappearance of the prozone phenomenon and detection of antigen levels typical of bacteria carrying plasmid pKT274 (data not shown). The $K1^-$ mutant derivatives $01:K^-$, C375 K^-, and $016:K^-$, which were isolated from their $K1^+$ parental strains by selection for resistance to K1-specific bacteriophages, showed in the same experiment no intracellular K1 antigen (data not shown). We conclude, therefore, that these mutants have a defect in K1 biosynthesis and not in the system for transmembrane transport of the polysaccharide.

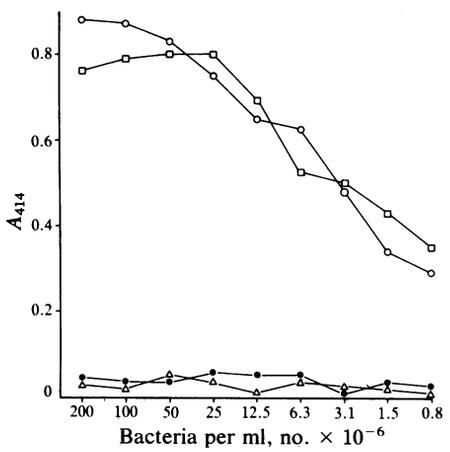

FIG. 1. K1 polysaccharide concentration-dependent reaction in ELISA of strains C375 K1 (circles), 016:K1 (squares), C375 K (filled circles), and 016 K^- (triangles).

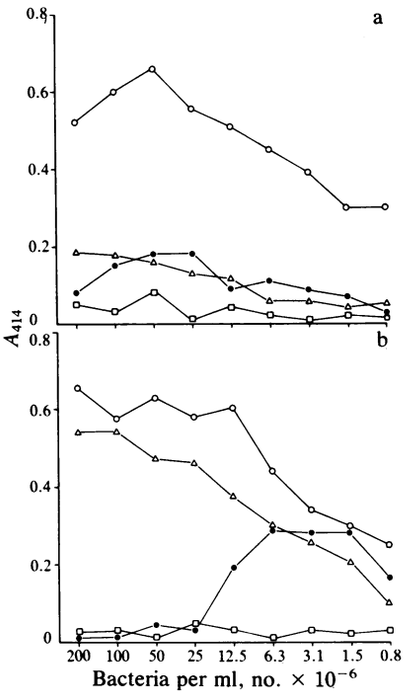

FIG. 2. K1 polysaccharide concentration-dependent reactions in ELISA of whole (*a*) or sonicated (*b*) *E. coli* K-12 LE392 derivatives carrying pKT274 (circles), pGB33 (triangles), pGB44 (filled circles), or no plasmid (squares).

Serological Properties of the Monoclonal Antibody 735D4. Both purified MBPS and Cetavlon (hexadecyltrimethylammonium bromide)-enriched K1 polysaccharide from *E. coli* D1737 were precipitated by the monoclonal antibody in immunodiffusion and immunoelectrophoresis experiments. For example, Fig. 3 shows immunoprecipitation of K1 antigen by the horse meningococcal polyclonal antibody (Fig. 3*a*) and the monoclonal antibody (Fig. 3*b*). Halo (precipitin) formation around colonies of strain C375 (Fig. 3*c*) but not around colonies of its derivative C375 K^- (Fig. 3*d*) was observed in monoclonal antibody-containing agar plates. Bacteria of meningococcus group B and *E. coli* K1 strains were directly agglutinated by 735D4 ascites fluid up to a dilution of 1:100, whereas no agglutination of bacteria of groups A or C menigococcus or of *E. coli* K^- strains was observed.

Comparison of the Immune Responses of BALB/c and NZB Mice to Group A, B, and C Meningococci. Although we were able to obtain a high-titer-IgG producing clone from the NZB mouse strain, we could not be certain whether this clone was induced by the immunization procedure or derived from a preexisting spontaneous autoimmune clone. To obtain information on this point and to determine whether the NZB model might be generally useful for the production of antibodies against weak polysaccharide antigens, we compared the IgG and IgM antipolysaccharide responses of NZB and BALB/c mice to immunization with group A, B, and C meningococci. Groups (10 per group) of either BALB/c or NZB

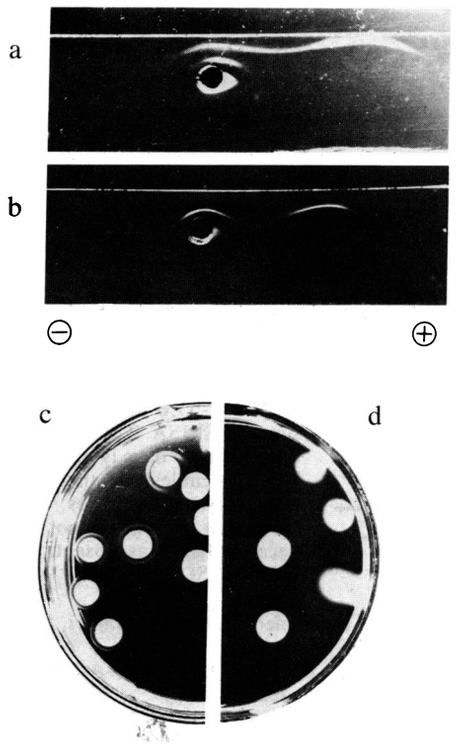

FIG. 3. (*a* and *b*) Immunoelectrophoresis with hexadecyltrimethylammonium bromide-enriched K1 polysaccharide of *E. coli* strain D1737 K1 and either horse meningococcal B antiserum (*a*) or monoclonal antibody 735D4 (*b*). Anode on the right. (*c* and *d*) Halo precipitin formation by monoclonal antibody 735D4 with C375 K1 (*c*) or C375 K^- (*d*).

mice were immunized with each type of bacteria as described in *Materials and Methods*. Five days after the primary immunization, BALB/c mice had developed an IgM response that did not change either in antibody titer or in immunoglobulin class after five subsequent injections (Fig. 4*a*). Treatment of sera with 2-mercaptoethanol totally abrogated the antibody titers. In contrast, NZB mice (Fig. 4*b*) exhibited only a weak IgM response after 5 days, but this was strengthened by the booster injections so that the total amount of antibody after the fifth booster injection equaled that of BALB/c mice. Treatment of NBZ sera with 2-mercaptoethanol eliminated only part of the antibody activity, indicating the presence of a considerable amount of IgG. Fig. 5 shows the immune response of BALB/c and NZB mice to meningococcus group A and C polysaccharides. Hyperimmunization with group C meningococci induced a strong immune response that was entirely IgG (protein A-binding; data not shown) in both types of mice (Fig. 5 *a* and *b*). Nevertheless, this response was 4 times higher in NZB mice than in BALB/c mice: an absorbance of 1.0 was produced by sera from the BALB/c group diluted ≈1:4000 (Fig. 5*a*) whereas an equal absorbance was produced by sera from the NZB group diluted 1:16,000 (Fig. 5*b*). The IgM values of both strains of mice at day 5 were the same. In contrast to the different responses of NZB and BALB/c mice to meningococcal B and C capsular antigens, no differences in responses to the strongly antigenic meningococcal A polysaccharide were seen (Fig. 5 *c* and *d*).

DISCUSSION

MBPS, which has been shown to be chemically and immunologically identical to K1 capsular polysaccharide of *E. coli*, is known for its weak immunogenicity (7). In contrast, group C and particularly group A meningococcal polysaccharides are better immunogens in humans (22). Group C meningococcal polysaccharide induces a short and weak IgM serum response in immunized mice (10). Although group B and C meningococcal polysaccharides are both polymers of Neu5Ac, they differ in the nature of the linkages between the monomeric units: namely, an $\alpha(2\rightarrow8)$ linkage in MBPS and an $\alpha(2\rightarrow9)$ linkage in group C polysaccharide. It is assumed that the poor immunogenicity of polymers of $\alpha(2\rightarrow8)$-linked Neu5Ac residues reflects the presence of Neu5Ac oligosaccharides having this linkage in gangliosides and glycoproteins of mammalian cells (21, 23). In an attempt to circumvent the poor immunogenicity of the K1/meningococcal B capsules, we substituted NZB for BALB/c mice. This approach was unconventional in terms of current hybridoma technology but seemed promising from a theoretical point of view. That is, we anticipated that a humoral immune response to weak polysaccharide antigenic determinants would be more easily provoked in the NZB mouse strain, which is known to exhibit hyperactivity of both its B-cell system (24) and its helper T-cell system (25). Indeed, an IgG response to MBPS was obtained in NZB but not BALB/c mice (Fig. 4), and an IgG2a monoclonal antibody was obtained. It may be that the antibody response of B-lymphocyte subsets to bacterial polysaccharide antigens belonging to the type 2 class of thymus-independent antigens (26, 27) is enhanced in B-cell hyperreactive NZB mice and/or that the usual activation of specific suppressor T cells by bacterial capsular polysaccharides (1) is altered in NZB mice.

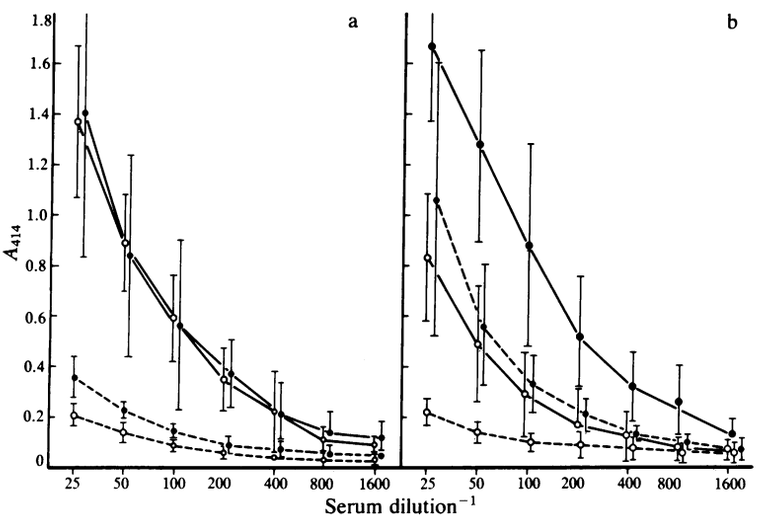

FIG. 4. Immune response to meningococcus B polysaccharide of BALB/c (*a*) and NZB mice (*b*) as measured by ELISA. The immune response after 5 days is shown by open circles, whereas the immune response after 5 weeks is shown by closed circles. Dotted lines indicate sera treated with 2-mercaptoethanol. Mean values ± SD are shown.

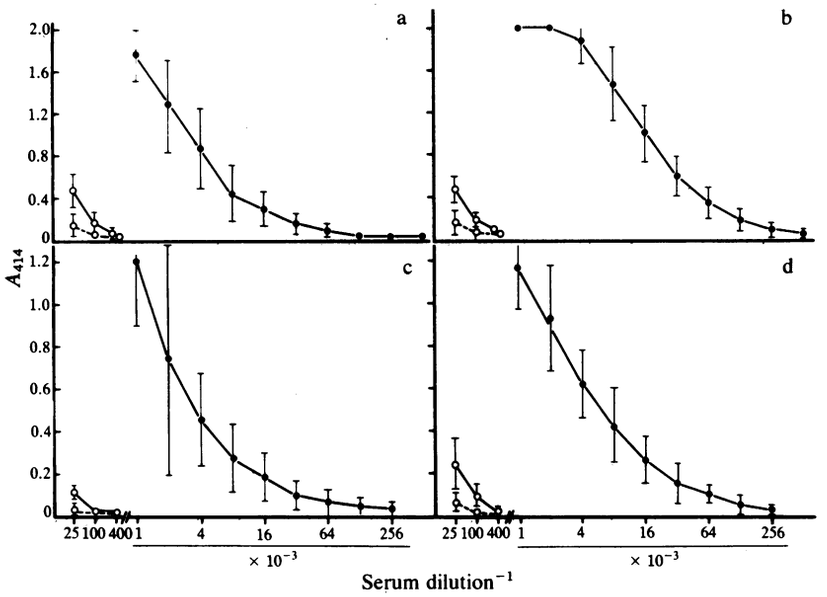

FIG. 5. Immune response to meningococcal C polysaccharide in BALB/c mice (*a*) and in NZB mice (*b*) and to meningococcal A polysaccharide in BALB/c mice (*c*) and in NZB mice (*d*) as measured in ELISA. The immune response after 5 days is represented by open circles and that after 5 weeks, by closed circles. Curves for IgM at 5 weeks are not given, because IgM was absent. Mean values ± SD are shown.

The production of IgM-type monoclonal antibodies against the K1 antigen was recently reported by two groups (28, 29). The antibody described here, being of the IgG-type, hence has superior properties—e.g., giving high-titer ascites fluids, binding to protein A (which simplifies purification and allows protein A serological procedures), ease of labeling, high stability to freezing and thawing, efficient antigen precipitation, and penetration of and diffusion within tissues. This antibody is an important addition to the analytical tools available for the study of the structure and function of the *E. coli* K1 and meningococcal B capsules.

Part of this work was done in fulfillment of the requirements of the University of Mainz for the M.D. thesis of M.F. This work was supported by the Bundesministerium für Forschung und Technologie, Bonn (Grant 01ZR042), and the Swiss National Science Foundation. G.J.B. gratefully acknowledges receipt of a postdoctoral fellowship from the European Molecular Biology Organization.

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