INFECTION AND IMMUNITY, Mar. 1996, p. 818–824 Vol. 64, No. 3
0019-9567/96/$04.00+0

Anti-Hsp65 Antibodies Recognize M Proteins of Group A Streptococci

ANTHONY QUINN,[1]† THOMAS M. SHINNICK,[2] AND MADELEINE W. CUNNINGHAM[1]*

Department of Microbiology and Immunology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73190,[1] and Division of Bacterial and Mycotic Diseases, National Center for Infectious Diseases, Centers for Disease Control, Atlanta, Georgia 30333[2]

Received 12 October 1995/Returned for modification 16 November 1995/Accepted 7 December 1995

Group A streptococcal M protein and the mycobacterial heat shock protein, hsp65, are strong bacterial immunogens that have been linked to arthritis and autoimmunity. Recent evidence has shown that streptococcal arthritis and adjuvant arthritis may be related to epitopes shared between group A streptococci and hsp65. We investigated the possibility that immunological similarities were shared between streptococcal M protein and hsp65. Antibodies against the 65-kDa heat shock protein of *Mycobacterium tuberculosis* were tested for reactivity with group A streptococci and purified recombinant M proteins (rM5 and rM6). Rabbit polyclonal anti-hsp65 serum was highly reactive with M type 5 *Streptococcus pyogenes* and rM5 and rM6 proteins in an enzyme-linked immunosorbent assay (ELISA). A mouse anti-hsp65 monoclonal antibody (MAb), IIC8, reacted with streptococcal M types 5, 6, 19, 24, and 49 in an ELISA but showed no reactivity with an isogenic streptococcal mutant which did not express M protein. Anti-hsp65 MAb IIC8 recognized rM5 and rM6 proteins in the ELISA, and MAbs IIC8 and IIH9 reacted strongly with rM6 protein in Western immunoblots. The binding of M protein by anti-hsp65 MAbs was shown to be inhibited by both hsp65 and M protein. These data show that anti-hsp65 antibodies recognize streptococcal M proteins.

Acute rheumatic fever (ARF) is an inflammatory disease thought to be mediated by autoimmune mechanisms initiated by a group A streptococcal infection (29, 32). Infection with the group A streptococcus induces the production of antibodies to several streptococcal antigens, including the major virulence factor, the cell-wall-associated streptococcal M protein (17, 33). There are more than 80 different serotypes of M protein, a molecule composed of two α-helical polypeptide chains organized into a coiled-coil fibril structure (17, 35, 37, 38). A host immune response that results in the development of opsonic antibodies to the M protein is critical in providing protection against the homologous streptococcal serotype. However, antibodies to the streptococcal M protein have also been shown to cross-react with various host tissue antigens (11, 12, 14). It has been suggested that antibodies to the M protein may play a role in the pathogenesis of rheumatic arthritis (2).

Heat shock proteins (hsps) are highly conserved proteins (54) that are often the immunodominant antigens recognized in bacterial, fungal, and parasitic infections. hsps are therefore capable of inducing strong humoral and cellular immune responses in mammals (7, 16, 22, 36, 42). However, because Hsps are also expressed in mammalian tissues (27), they could provide a mechanism by which infectious agents might induce autoimmune disease in susceptible individuals (28, 50). Hsps have been implicated in the pathogenesis of several autoimmune diseases, including adjuvant-induced arthritis in rats (5, 8, 36). Adjuvant arthritis is a disease observed in susceptible rats immunized with complete Freund's adjuvant which contains components of heat-killed *Mycobacterium tuberculosis* (8). It was shown that T cells reactive with the 65-kDa hsp of mycobacteria (hsp65) were capable of transferring arthritis to naive animals (25). Epitope mapping of arthritogenic T-cell clones recovered from rats immunized with mycobacteria has led to the identification of an hsp65 sequence that cross-reacts with a self antigen in joint cartilage (24, 51).

Severe polyarthritis can also be induced in Lewis rats by intraperitoneal injection of streptococcal cells or cell walls (13, 18). It was discovered that prior vaccination with hsp65 protected animals from the subsequent induction of streptococcal cell wall arthritis as well as adjuvant arthritis (49). These data suggested that immunological similarities were shared between the group A streptococcus and mycobacterial hsp65 and that these epitopes might be involved in the pathogenesis of arthritis. In the present study we used polyclonal and monoclonal antibody probes produced against the mycobacterial hsp65 to determine whether they also reacted with the group A streptococcus. The data strongly suggest that immunological similarities exist between hsp65 and the streptococcal M protein. Furthermore, the study shows that anti-hsp65 antibody can cross-react with type II collagen. Type II collagen is an autoantigen found in mammalian joint tissue and has been shown to induce autoimmune arthritis in mice (46–48).

MATERIALS AND METHODS

Antibodies. Rabbit polyclonal antiserum was produced against the 65-kDa heat shock protein of *M. tuberculosis* and the 60-kDa heat shock protein of *Legionella pneumophila* (39). Alum hydroxide was used as the adjuvant during immunization of the animals. Four murine monoclonal antibodies (MAbs), IIC8, (immunoglobulin G2b [IgG2b]), IIH9 (IgG2a), IVD8 (IgG1), and IIIE9 (IgG1), which recognize the 65-kDa mycobacterial heat shock protein were produced by the fusion of splenocytes from BALB/c mice immunized with extracts of *Mycobacterium leprae* (21). Anti-hsp65 MAb ML30 (IgG1) originated in the laboratory of J. Ivanyi (55). These MAbs have been extensively characterized serologically by the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (1, 6, 55). The MAbs were supplied as lyophilized ascites fluids by the World Health Organization. The MAbs were diluted 1:1 in Hanks balanced salt solution before lyophilization. Upon receipt, the MAbs were re-

* Corresponding author. Mailing address: Department of Microbiology and Immunology, University of Oklahoma Health Sciences Center, 940 Stanton L. Young Blvd., Oklahoma City, OK 73104. Phone: (405) 271-3128. Fax: (405) 271-3117. Electronic mail address: madeleine-cunningham@uokhsc.edu.

† Present address: Department of Microbiology and Molecular Genetics, University of California at Los Angeles, Los Angeles, CA 90024.

constituted in phosphate-buffered saline (PBS) as directed and diluted 1:100 in PBS. This dilution had been used previously in the characterization of these MAb fluids from the World Health Organization (1, 6, 21, 55).

ELISA. Fifty microliters each of recombinant type 5 M protein (rM5) (a gift from J. B. Dale, Veterans Administration Medical Center, Memphis, Tenn.), recombinant type 6 M protein (rM6) (a gift from V. Fischetti, The Rockefeller University, New York, N.Y.), *N*-acetylglucosamine conjugated to bovine serum albumin (BSA) (50:1) (a gift from A. Shikhman, University of Oklahoma Health Science Center, Oklahoma City, Okla.), mouse laminin (Sigma Chemical Co., St. Louis, Mo.), and bovine type II collagen (Chemicon, Temecula, Calif.) was coated onto Immulon-4 96-well microtiter plates (Dynatech, Chantilly, Va.) at 10 μg/ml overnight at 4°C. Plates were washed three times with PBS–0.05% Tween 20 and blocked for 1 h at 37°C with 1% BSA in wash buffer (Sigma). Fifty microliters of diluted rabbit antisera was then added in duplicate to wells and incubated overnight at 4°C. Plates were washed, and 50 microliters of goat anti-rabbit-Ig conjugated to alkaline phosphatase (Sigma) was reacted with the primary antibody, while *p*-nitrophenylphosphate (Sigma) was used as the enzyme substrate. After 30 min the A_{410} in each well was determined by using an enzyme-linked immunosorbent assay (ELISA) plate reader (Dynatech). Titers were reported as the reciprocal of the serum dilution which would give an absorbance of 0.200. MAbs were diluted 1:100 with buffer containing 1% BSA and tested in the ELISA described above. Goat anti-mouse IgG-biotin conjugate and avidin-alkaline phosphatase conjugate (Sigma) were added sequentially to wells and incubated for 2 h at 37°C to detect primary antibody. Values were reported as the A_{410}. All determinations were performed in duplicate. The determinations were repeated three times, and the variance between duplicate tests was less than 10%. A positive or significant titer was considered to be a fourfold increase over the control titer, which is a standard conventional limit.

Streptococcal cell ELISA. Microtiter plates used in the streptococcal cell ELISA were prepared as previously described (53). Briefly, *Streptococcus pyogenes* cells, including M protein serotypes 5, 6, 19, 24, and 49 and an isogenic mutant of the type 6 strain in which the gene for the M protein has been insertionally inactivated (a gift from Susan Hollingshead, University of Alabama, Birmingham) were glutaraldehyde fixed to 96-well, polyvinylchloride plates (Dynatech). The ELISA was performed as previously described (12). All tests were performed in duplicate, and experiments were repeated several times. All reagents were used in 50-μl aliquots.

Competitive Inhibition ELISA. The procedure for the competitive inhibition ELISA has been previously described (12). Antibodies diluted 1:100 in PBS were preincubated 1:1 with recombinant hsp65 or PepM protein (500 μg/ml) for 1 h at 37°C and then overnight at 4°C before being reacted in the ELISA. The absorbance value of antibody diluted in buffer was considered as 100% reactivity. Recombinant mycobacterial hsp65 was produced from plasmid pTB12 (41) propagated in *Escherichia coli*. *E. coli*(pTB12) was grown at 37°C in Luria broth containing 33 μg of ampicillin per ml. The cells were harvested by centrifugation and resuspended in PBS; this was followed by two cycles of freezing in liquid nitrogen and rapid thawing (6). The preparation was then centrifuged, and the supernatant containing the 65-kDa heat shock protein was recovered. Pepsin-extracted type 5 M protein (PepM5) was prepared as previously described (3, 10). Percent inhibition was calculated according to the following formula: $100 \times [(A_{410}$ of well with MAb plus buffer $- A_{410}$ of well with MAb plus inhibitor$)/A_{410}$ of well with MAb plus buffer].

Immunoblots. Western immunoblotting was performed as previously described (12). Recombinant streptococcal M proteins and type II collagen were separated by electrophoresis in 10% reducing polyacrylamide gels with sodium dodecyl sulfate (SDS-PAGE). Proteins were electrophoretically transferred to nitrocellulose membranes and blocked for 1 h with 3% Tween 20 in PBS. Blots were then reacted with MAbs (1:100) in 0.5% Tween 20 in PBS overnight at 4°C. After being washed with 0.5% Tween 20, blots were reacted with goat anti-rabbit Ig–peroxidase (1:200; Sigma) or goat anti-mouse Ig–peroxidase (1:200; Sigma) for 1 h at room temperature. After the blots were washed, H_2O_2 and 4-chloronaphthol (Sigma) were mixed and added to the blots to detect positive reactions.

RESULTS

Reaction of rabbit Anti-hsp65 with group A streptococcal antigens. Rabbit anti-hsp65 serum was tested for reactivity with group A streptococcal cells in the ELISA. The anti-hsp65 serum had a titer of 180,000 against the type 5 streptococci (Table 1). This was a 200-fold increase over the titer observed when the rabbit preimmune serum was reacted with the type 5 streptococcal cells (Table 1). The anti-hsp65 serum also showed strong reactivity with group A streptococcal serotypes 6, 19, 24, and 49 when compared with the levels of reactivity of the preimmune serum (Table 1). A rabbit antiserum produced against the 60-kDa hsp of *L. pneumophila* (Rb231) was used as a control in these experiments. Although it had three times higher reactivity than the preimmune control, the anti-hsp60 serum was not very reactive with streptococci in the ELISA when compared with the strong reactivity of the anti-hsp65 serum (Table 1). Values which were a fourfold increase over that for the preimmune control were considered to be significant, while values less than a fourfold increase were considered not significant. The values of the anti-hsp65 serum reactivity ranged from a 200-fold increase (M type 5) to a 27-fold increase (M type 24) to a 13- to 14-fold increase (M types 6, 19, and 49). Similar results were obtained in three separate experiments each performed in duplicate. In duplicate tests the variance was less than 10%.

TABLE 1. Reaction of rabbit anti-hsp65 with group A streptococci and streptococcal M proteins

M type[a]	ELISA titer[b]		
	Preimmune serum	Anti-*M. bovis* hsp65	Anti-*L. pneumophila* hsp60
M5	900	180,000	2,000
M6	3,900	58,000	5,900
M19	3,900	58,000	11,000
M24	1,700	47,000	6,000
M49	4,500	58,000	11,500
rM5	2,300	86,000	3,900
rM6	660	27,000	800
N-acetylglucosamine-BSA	240	1,700	<100

[a] M protein serotypes of group A streptococci glutaraldehyde fixed to microtiter plates.
[b] Titer in ELISA was determined as described in Materials and Methods.

In previous experiments, we found that the anti-hsp60 serum (Rb231) reacted with what appeared to be a streptococcal homolog to hsp60 (unpublished data). When lysates of group A streptococci incubated at 37 and 42°C for various times were tested in the immunoblot assay, the anti-hsp60 serum (RB231) reacted with a molecule with a molecular mass of approximately 60 kDa. This molecule was present in lysates of streptococci grown at 42°C but not those grown at 37°C. In the same experiments, the anti-hsp65 serum did not react with the 60-kDa band nor did it identify any new molecules in the lysates of heat-shocked streptococci. Thus the streptococcal hsp60 was immunologically similar to the hsp60 of *L. pneumophila* but was not recognized by the antiserum to hsp65 of mycobacteria.

The anti-hsp65 serum was then tested for reactivity with two immunodominant antigens associated with the streptococcal cell wall. The M protein is a surface-exposed streptococcal protein, and *N*-acetylglucosamine is the major component of the streptococcal cell wall carbohydrate (19). The anti-hsp65 serum reacted with two purified recombinant M proteins in the ELISA. The anti-hsp65 serum had titers of 86,000 and 27,000 with rM5 and rM6, respectively (Table 1). These anti-M protein titers were greatly increased over those of the preimmune or anti-hsp60 serum (Table 1). However, when tested in the ELISA for reactivity with *N*-acetylglucosamine the anti-hsp65 serum showed little reactivity with *N*-acetylglucosamine conjugated to BSA (Table 1). These data suggested that the anti-hsp65 serum may cross-react with M protein on the surface of group A streptococcal cells.

Reaction of anti-hsp65 MAbs with streptococcal M proteins. To determine if the M protein was involved in immunological cross-reaction between group A streptococci and the mycobacterial hsp65, we first tested an anti-hsp65 MAb, IIC8, for reactivity with streptococcal cells and purified recombinant M proteins. MAb IIC8, previously shown to recognize the hsp65 of both *M. tuberculosis* and *M. leprae* (6), was reacted with

TABLE 2. Reactivity of anti-hsp65 MAb IIC8 with group A streptococci and recombinant streptococcal M proteins

M type[a]	A_{410} in ELISA	
	MAb IIC8[b]	Control[c]
M5	0.642	0.170
M6	0.808	0.220
M6 (−)[d]	0.099	0.150
M19	1.317	0.160
M24	0.659	0.140
M49	1.695	0.140
rM5	0.488	0.097
rM6	1.380	0.051

[a] Group A streptococcal serotype.
[b] Anti-hsp65 MAb, 1:100.
[c] Normal mouse serum, 10 μg/ml.
[d] M protein-negative isogenic mutant.

group A streptococci in an ELISA. A normal mouse serum pool (Table 2) and anti-hsp65 MAb ML30 were used as controls. Absorbance values that were twice that of the control serum and ML30 were considered positive. In the ELISA, MAb IIC8 strongly reacted with streptococcal serotypes 5, 6, 19, 24, and 49 (Table 2). Although data are not shown in the table, anti-hsp65 MAb ML30 reacted with the whole streptococci at the same optical density level as the normal mouse serum shown in Table 2. The experiment for which results are shown in Table 2 was performed twice, and in duplicate tests the variance between assays was less than 10%. To further demonstrate that M protein was involved in the cross-reaction of the anti-hsp65 MAbs with streptococci, anti-hsp65 MAbs were tested for reactivity with an M protein-negative, isogenic mutant of the streptococcal type 6 strain D471. This mutant, which does not express the M protein, was fixed to plates at the same time as those strains which did express the protein. The anti-hsp65 MAb IIC8 showed no reactivity with the M protein-negative strain (Table 2). Thus, expression of M protein was necessary for anti-hsp65 MAb IIC8 to react with the group A streptococci.

Anti-hsp65 MAb IIC8 was shown to react with rM5 and rM6 proteins in the ELISA (Table 2). Anti-hsp65 MAb ML30 served as a baseline control for the anti-hsp65 MAb IIC8 and did not react with rM5 protein or with the rM6 protein at optical densities above 0.2. The mouse serum control showed no significant reaction (optical density, approximately 0.1) with any of the M proteins tested (Table 2). It should be noted that the anti-hsp65 MAbs used in this study were previously tested for recognition of *E. coli* products (6). No reactivity was detected with any *E. coli* protein, including GroEL. These findings support the conclusion that anti-hsp65 MAb IIC8 reacted with M protein.

To analyze further the cross-reactivity between hsp65 and M protein, two anti-hsp65 MAbs, IIC8 and IIH9, were tested for binding to rM6 protein in a Western immunoblot assay. The MAbs were diluted 1:100 in PBS-Tween containing 1% BSA before being reacted with M protein. This was the MAb dilution that we found was necessary to detect recombinant hsp65 (rhsp65) in the immunoblot assay using MAb IIC8 (data not shown) and previously used to show the reactivity of MAb IIH9 with hsp65 (27). Twenty micrograms of rM6 was separated by electrophoresis in a discontinuous polyacrylamide gel and transferred to a nitrocellulose membrane. Both MAbs IIC8 and IIH9 reacted with the rM6 protein in the Western immunoblot assay (Fig. 1). The bands detected by the anti-hsp65 antibodies were identical to those seen in the protein

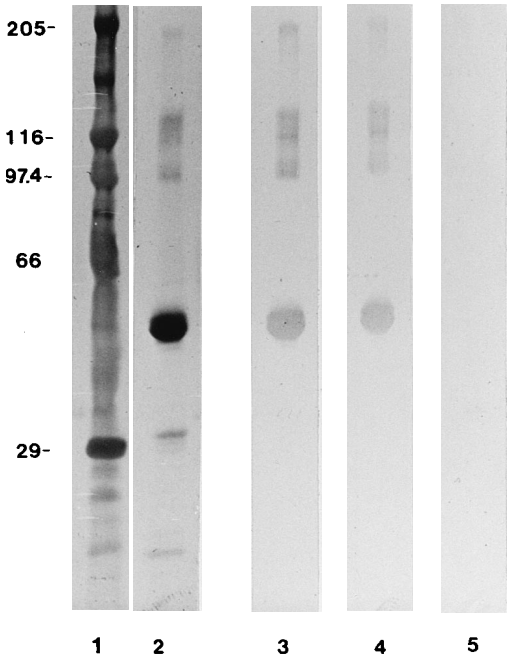

FIG. 1. Reaction of anti-hsp65 MAbs with rM6 in the Western immunoblot. Lane 1, molecular weight standards stained with Coomassie blue; lane 2, rM6 stained with Coomassie blue; lane 3, anti-hsp65 MAb IIC8 (1:100) reacted with rM6 protein; lane 4, anti-hsp65 MAb IIH9 (1:100) reacted with rM6 protein; lane 5, MAb control reacted with rM6 protein. Anti-hsp65 MAbs IIC8 and IIH9 reacted strongly with rM6 protein (lanes 3 and 4). The MAb control (lane 5) was negative. A strong reaction was observed with the rM6 protein near 60 kDa. The higher-molecular weight protein bands seen in the blot are aggregates characteristically seen in rM6 protein preparations.

stain (Fig. 1) and the same as those detected by other anti-M protein antibodies (data not shown). A conjugate control (not shown) and a mouse IgG MAb control (Fig. 1) were negative in the immunoblot. Therefore, the immunoblot results supported the ELISA data, suggesting that the streptococcal M protein and the mycobacterial hsp65 shared immunological epitopes.

To confirm that anti-hsp65 MAbs crossreacted with streptococcal M proteins, a competitive inhibition ELISA was performed. Three anti-hsp65 MAbs, IIC8, IIIE9, and IVD8, were preincubated with preparations of rhsp65 protein before reaction with streptococcal rM5 protein in the ELISA (Table 3). The rM5 was plated onto ELISA plates at 50 μl of a 10 μg/ml solution. The antigen and antibody were mixed 1:1 together before incubation at 37°C for 30 min and overnight at 4°C. The mixture was added to the ELISA plate at 50 μl, and the ELISA

TABLE 3. Inhibition of the binding of anti-hsp65 MAbs to rM5 protein

Antibody	Absorbance[a]	% Inhibition[b]	
		rhsp65 (*E. coli* lysate)	Pep M5 (500 μg/ml)
IVD8[c]	1.717	50.4	63.9
IIIE9[c]	0.606	57.3	58.6
IIC8[c]	0.488	33.6	44.3
49.8.9[d]	1.428	0	52.2

[a] Absorbance values of wells without inhibitor.
[b] Percent inhibition was determined as described in Materials and Methods.
[c] Anti-hsp65 MAb.
[d] Antistreptococcal MAb that reacts with type 5 M protein.

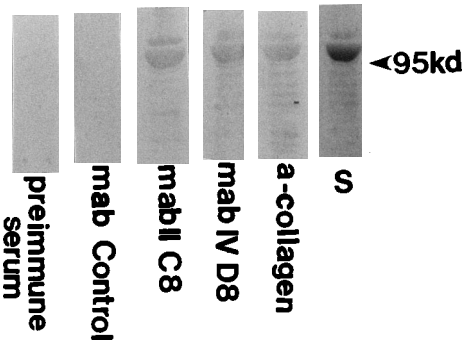

FIG. 2. Reaction of anti-hsp65 MAbs with purified type II collagen in the Western immunoblot. Purified type II collagen reacted with Coomassie blue stain (S) shows the 100-kDa protein band of collagen blotted after SDS-PAGE. a-collagen shows the rabbit anti-type II collagen reacted with type II collagen. Anti-hsp65 MAb IIC8 (1:100) and anti-hsp65 MAb IVD8 (1:100) are shown reacted with type II collagen. A MAb control and a preimmune serum were negative when reacted with the collagen as shown. Collagen is composed of three chains of 100 kDa each, and the 100-kDa band was as expected. The anticollagen serum and the anti-hsp MAbs reacted with the major protein band at 100 kDa. The amino acid analysis provided by Chemicon of the purified protein revealed that it was consistent with collagen.

procedure was performed as described in Materials and Methods. The rhsp65 inhibited (33 to 57% inhibition) the reaction of the anti-hsp65 MAbs with streptococcal rM5 protein in the ELISA (Table 3). In additional experiments, a pepsin cleavage fragment of streptococcal type 5 M protein (PepM5) was used to inhibit the binding of the anti-hsp65 MAbs to recombinant M protein. Streptococcal PepM5 inhibited the reactivity of anti-hsp65 MAbs IVD8, IIIE9, and IIC8 with rM5 protein by 63.9, 58.6, and 44.3%, respectively. A control antistreptococcal MAb, 49.8.9, known to react with type 5 M protein was not inhibited by rhsp65 lysate but was inhibited by PepM5 protein at a concentration of 500 μg/ml (Table 3). Since PepM5 is prepared by treating streptococci with the enzyme pepsin, the observed inhibitions can be attributed to M protein epitopes but cannot be due to *E. coli* contaminants. These results demonstrate that the reactivity of the anti-hsp65 MAbs IVD8, IIIE9, and IIC8 with M protein was inhibited by both hsp65 and M protein.

Reaction of anti-hsp65 antibodies with type II collagen. Type II collagen is a protein found in joint synovium and is capable of inducing autoimmune arthritis in mice (48). Since hsp65 is suspected of inducing immunoreactivity to autoantigens found in joint tissue, both polyclonal antisera and MAbs to hsp65 were tested for reactivity with type II collagen in the ELISA and Western immunoblot assay. The rabbit anti-hsp65 serum reacted strongly with the type II collagen in the ELISA, with a titer against collagen of 10,000, while the preimmune serum and the anti-hsp60 serum each had a titer of only 700. To demonstrate that epitopes were shared between hsp65 and type II collagen, anti-hsp65 MAbs IIC8 and IVD8 were tested for reactivity with purified type II collagen in a Western immunoblot assay. Twenty micrograms of type II collagen was separated in an SDS–10% polyacrylamide gel before being transferred to a nitrocellulose membrane. A protein band which migrated at 100 kDa reacted with the rabbit anti-type II collagen antiserum (Fig. 2) but not with a normal rabbit serum pool (data not shown). We found that both anti-hsp65 MAb IIC8 and MAb IVD8 reacted with type II collagen in the blot (Fig. 2) while a normal mouse serum pool and an ascites fluid containing mouse IgG showed no reaction with the collagen in the blot. The rabbit anti-hsp65 serum was also reactive with type II collagen in the Western immunoblot but showed no reactivity with type X collagen (data not shown). Therefore, we found that antibodies to mycobacterial hsp65 cross-reacted with M protein and also demonstrated cross-reactivity with type II collagen. Because antibodies to streptococci were reported to react with antigens found in the joints (2), a rabbit antiserum produced against type 5 group A streptococci was also tested in the ELISA for reactivity with type II collagen. The anti-type 5 antiserum displayed a titer of 8,640, a greater than 10-fold increase over that of the nonimmune serum.

TABLE 4. Inhibition of anti-hsp65 MAb binding to rM5 by type II collagen

MAb[a]	OD[b] in buffer	% Inhibition[c]	
		Collagen (100 μg/ml)	Laminin (100 μg/ml)
IVD8	0.439	71.3	0.0
IIC8	0.421	74.5	12.7

[a] Anti-hsp65 MAb.
[b] OD, optical density at 410 nm.
[c] Percent inhibition was determined as described in Materials and Methods.

Inhibition of antibody binding to M protein or to an hsp65 peptide by type II collagen. To determine if the binding of M protein by anti-hsp65 MAbs could be inhibited by type II collagen, MAbs IIC8 and IVD8 were preincubated with 100 μg of type II collagen per ml before being reacted with streptococcal rM5 protein in the ELISA. Type II collagen inhibited the binding of anti-hsp65 MAbs IIC8 and IVD8 to M5 protein by 74.5 and 71.3%, respectively (Table 4). The MAbs were not inhibited by laminin, which was tested as a control antigen (Table 4).

Additionally, an inhibition assay was performed in which MAb IIC8 was preincubated with type II collagen prior to reaction with the hsp65 synthetic peptide VYEDLLAAG VAD. This peptide contains the *M. tuberculosis* hsp65 epitope recognized by MAb IIC8. Type II collagen inhibited the binding of MAb IIC8 to the hsp65 peptide in a dose-dependent fashion (Fig. 3). Type II collagen inhibited the reaction of MAb IIC8 with the hsp65 peptide by 60% at 100 μg of collagen per ml and by 30% with as little as 20 μg of collagen per ml (Fig. 3). Ten micrograms of the autologous peptide per ml inhibited the binding of IIC8 to the hsp65 peptide by 100% (data not shown). Laminin did not inhibit MAb IIC8 at any concentration tested (Fig. 3). The strong reaction of anti-hsp65 MAb IIC8 with collagen further supports the hypothesis that some anti-hsp65 antibodies, in addition to binding streptococcal M proteins, can also recognize type II collagen.

DISCUSSION

Epitopes shared between immunodominant antigens of infectious agents may be important in the development of immunity against diverse pathogens (9, 31, 42). During infection, microbial heat shock proteins may provide the immune system with evolutionarily conserved epitopes which are capable of inducing cross-reactive immune responses. These cross-reactive responses could then provide protection against a number of microbial pathogens. However, heat shock proteins may also be involved in the induction of autoimmune responses which may become pathogenic in humans (30). This hypothesis is supported by the reported isolation of an hsp65-reactive T-cell clone from the synovial fluid of a patient with rheumatoid arthritis (20, 40).

Arthritis occurs in approximately 75% of the patients with

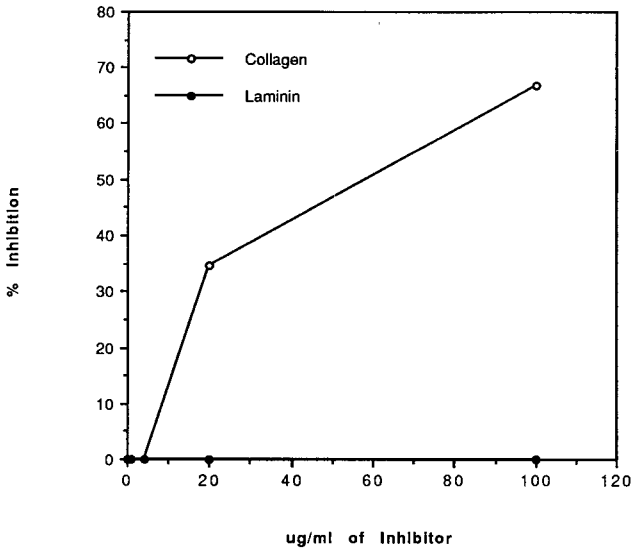

FIG. 3. Type II collagen inhibits binding of anti-hsp65 MAb IIC8 to hsp65 peptide VYEDLLAAGVAD. MAb IIC8 (1:200) was preincubated with various concentrations of type II collagen or laminin before being reacted with the synthetic mycobacterial hsp65 peptide VYEDLLAAGVAD in the ELISA. Percent inhibitions were calculated as described in Materials and Methods.

ARF (26). Although the arthritis is often severe, with swelling and edema of the large joints, it is usually self-limiting and occurs without erosion of the joint surface or other long-term damaging effects. The mechanism of disease induction in ARF is uncertain. However, it is known that rheumatic arthritis normally occurs shortly after a streptococcal infection and is associated with a rising titer of antistreptococcal antibodies (32). It has been reported that streptococcal M proteins may share epitopes with antigens present in host joint tissue (2). Baird et al. demonstrated that rabbit antisera to Pep M protein could cross-react with articular cartilage and synovial tissue (2). Such cross-reactive antibodies were also found in the serum of rheumatic fever patients with arthritis, thus suggesting a role for M protein in the pathogenesis of rheumatic arthritis. It has been previously observed that animals immunized with mycobacterial hsp65 were protected from the subsequent induction of arthritis by streptococci (49) and that arthritogenic, streptococcal cell wall-specific T-cell lines proliferated in response to sonicates of *M. tuberculosis* (15). These data imply that there is antigenic cross-reactivity between group A streptococci and hsp65. While others have suggested that a streptococcal cell wall antigen shares epitopes with hsp65 (15, 34), no homolog to the mycobacterial hsp65 has been reported in streptococci. Here we report that both polyclonal antibodies and MAbs to the mycobacterial hsp65 cross-react with at least two serotypes of streptococcal M protein. Although the anti-hsp65 antibodies reacted strongly with different forms of streptococcal M proteins and were inhibited by M protein, it is possible that an *S. pyogenes* hsp65 or some other antigen might also play a role in the immunological cross-reaction between streptococcal cells and mycobacterial hsp65.

Since the bacterial antigens M protein and hsp65 are associated with autoimmunity (11, 12, 51, 52) and are thought to be involved in arthritis induction, it is possible that epitopes shared between hsp65 and M proteins may be capable of inducing autoimmune responses that participate in the pathogenesis of arthritis. Molecular mimicry, or the sharing of immunological epitopes between host and pathogen, has been proposed as a mechanism which may lead to the pathogenesis of autoimmune disease (9). In the case of arthritis, proteoglycan (51) and vimentin (2) have been identified as candidate autoantigens which may cross-react with hsp65 and M protein, respectively. Type II collagen is another autoantigen found in joint tissue which has been shown to activate immune responses capable of inducing arthritis in certain strains of mice (23, 46–48). In addition, it has been reported that the induction of tolerance to collagen attenuates mycobacterium-induced adjuvant arthritis (56) and that the severity of arthritis induced by collagen immunization can be attenuated by administration of hsp65 (5). Moreover, it has been clearly demonstrated that antibodies to type II collagen are capable of inducing arthritis and joint inflammation in mice (23, 43, 44, 46). Arthritis seen in rheumatic fever may be severe, but it is generally self-limiting and usually resolves without permanent damage to the joint (26, 32). Immunoglobulins and complement in the synovial fluid in rheumatic fever have been reported (45); however, whether the streptococcal M protein plays a direct role in the development of rheumatic arthritis has not been proven.

In this study, we demonstrated that anti-hsp65 antibodies cross-react with streptococcal M protein in the ELISA and Western immunoblot assay and also recognize the autoantigen type II collagen. We showed that the hsp65 amino acid sequence recognized by MAb IIC8 blocked the reaction of that antibody with M protein. The M protein amino acid sequence has been divided into the A, B, and C repeat regions as described by Fischetti (17). Amino acid sequence homology between the IIC8 hsp65 peptide sequence VY**ED**L**L**AAG**V**AD and M5 protein was found in the M5 sequence EN**E**G**L**K TENEGL, which is located in the A repeat region (residues 128 to 139) and is found again in the B repeat region (residues 213 to 222) of M5 protein. The boldface amino acids are the sites of identity between M5 protein and the IIC8 hsp65 sequence. When the mycobacterial hsp65 sequence recognized by MAb IIH9 (AAG**A**NPL**GLKR**GIEKA) (1) was compared with M protein, homology was found with the sequences ISD**A**SRK**GLRR**DLDAS (residues 301 to 316) and ISE**A**S RK**GLRR**DLDAS (residues 370 to 385). Both of these M5 sequences are located in the C repeat region near a site described as the class I M protein epitope associated with rheumatogenic serotypes (4). Anti-hsp65 MAbs IIIE9 and IVD8 are thought to recognize the same epitope on the hsp65 of *M. leprae*. The hsp65 amino acid binding sequence reported for MAb IIIE9 (K**LK**LTG**DE**A) (1) was also compared with the type 5 M protein. Homology was found with the M5 amino acid sequence GT**LK**KIL**DE**T, which is located twice in the B repeat region of type 5 M protein (residues 153 to 161 and 178 to 186). Interestingly, amino acids at positions 4, 8, and 9 of the IIIE9 hsp65 peptide sequence (K, D, and E) have identity with the homologous M protein sequence and have been identified as residues critical to the binding of MAb IIIE9 (1). These similarities may play a role in the binding of the anti-hsp65 MAbs to M protein. On the other hand, conformations not recognized by sequence homology may also play a role in the cross-reactivity.

Repeated regions within the M proteins may greatly enhance the host response against a particular epitope within a protein and also enhance the binding of cross-reactive antibodies. From our point of view, the homologies between the two proteins may be responsible for the immunological mimicry observed in our study. The similarities between the two proteins, either structural or conformational, may influence the outcome of adjuvant arthritis, as previously reported (15). In addition, the potentially strong adjuvant properties of the

mycobacterial hsp65-peptidoglycan complex may have similarities with the group A streptococcal peptidoglycan-polysaccharide-M protein complex. Further studies are needed to completely understand the relationship of complex bacterial antigens and development of autoimmune diseases such as arthritis. Finally, heat shock proteins, because of their conserved sequences or epitopes, are thought to be ideal targets for the immune system. Immune responses directed at strongly immunogenic epitopes shared among microbial pathogens build a first line of defense which can easily be formed and maintained in the host. However, if a shared epitope is also immunologically cross-reactive with host tissue, then autoimmunity could be induced by successive exposure to a number of pathogens. Therefore, mimicry between host and pathogens as well as mimicry between pathogens themselves may play an important role in the balance between protective immunity and autoimmunity.

ACKNOWLEDGMENTS

We thank Susan Hollingshead for the M6 protein gene deletion mutant, Vincent Fischetti for purified recombinant M6 protein, James Dale for purified recombinant M5 protein, Alexander Shikhman for *N*-acetylglucosamine-BSA, Carol Crossley and Laurie Hynes for excellent technical assistance, and Mary Patterson for excellent secretarial assistance.

This work was supported by a grant from the National Heart, Lung, and Blood Institute (HL35280).

REFERENCES

1. **Anderson, D. C., M. E. Barry, and T. M. Buchanan.** 1988. Exact definition of species-specific and cross-reactive epitopes of the 65-kilodalton protein of *Mycobacterium leprae* using synthetic peptides. J. Immunol. **141:**607–613.
2. **Baird, R. W., M. S. Bronze, K. Werner, H. R. Hill, L. G. Veasy, and J. B. Dale.** 1991. Epitopes of group A streptococcal M protein shared with antigens of articular cartilage and synovium. J. Immunol. **146:**3132–3137.
3. **Beachey, E. H., G. L. Campbell, and I. Ofek.** 1974. Peptic digestion of streptococcal M protein. II. Extraction of M antigen from group A streptococci with pepsin. Infect. Immun. **9:**891–896.
4. **Bessen, D., K. F. Jones, and V. A. Fischetti.** 1989. Evidence for two distinct classes of streptococcal M protein and their relationship to rheumatic fever. J. Exp. Med. **169:**269–283.
5. **Billingham, M. E. J., S. Carney, R. Butter, and M. J. Colston.** 1990. A mycobacterial 65-kDa heat shock protein induces anti-specific suppression of adjuvant arthritis, but is not itself arthritogenic. J. Exp. Med. **171:**339–344.
6. **Buchanan, T. M., H. Nomaguchi, D. C. Anderson, R. A. Young, T. P. Gillis, W. J. Britton, J. Ivanyi, A. H. J. Kolk, O. Closs, B. R. Bloom, and V. Mehra.** 1987. Characterization of antibody-reactive epitopes on the 65-kilodalton protein of *Mycobacterium leprae*. Infect. Immun. **55:**1000–1003.
7. **Burnie, J. P., and R. C. Matthews.** 1991. Heat shock protein 88 and *Aspergillus* infection. J. Clin. Microbiol. **29:**2099–2106.
8. **Cohen, I. R.** 1991. Autoimmunity to chaperonins in the pathogenesis of arthritis and diabetes. Annu. Rev. Immunol. **9:**567–589.
9. **Cunningham, M. W., S. M. Antone, J. M. Gulizia, B. M. McManus, V. A. Fischetti, and C. J. Gauntt.** 1992. Cytotoxic and viral neutralizing antibodies crossreact with streptococcal M protein, enteroviruses, and human cardiac myosin. Proc. Natl. Acad. Sci. USA **89:**1320–1324.
10. **Cunningham, M. W., and E. H. Beachey.** 1974. Peptic digestion of streptococcal M protein. I. Effect of digestion at suboptimal pH upon the biological and immunochemical properties of purified M protein extracts. Infect. Immun. **9:**244–248.
11. **Cunningham, M. W., J. M. McCormack, L. R. Talaber, J. B. Harley, E. M. Ayoub, R. S. Muneer, L. T. Chun, and D. V. Reddy.** 1988. Human monoclonal antibodies reactive with antigens of the group A streptococcus and human heart. J. Immunol. **141:**2760–2766.
12. **Cunningham, M. W., and R. W. Swerlick.** 1986. Polyspecificity of anti-streptococcal murine monoclonal antibodies and their implications in autoimmunity. J. Exp. Med. **164:**998–1012.
13. **Cromartie, W. J., J. G. Craddock, J. H. Schwab, S. K. Anderle, and C. H. Yang.** 1977. Arthritis in rats after systemic injection of streptococcal cells or cell walls. J. Exp. Med. **146:**1585–1602.
14. **Dale, J. B., and E. H. Beachey.** 1985. Epitopes of streptococcal M proteins shared with cardiac myosin. J. Exp. Med. **162:**583–591.
15. **Dejoy, S. Q., K. M. Ferguson, T. M. Sapp, J. B. Zabriskie, A. L. Oronsky, and S. S. Kerwar.** 1989. Streptococcal cell wall arthritis. Passive transfer of disease with a T cell line and crossreactivity of streptococcal cell wall antigens with Mycobacterium tuberculosis. J. Exp. Med. **170:**369–382.
16. **Esaguy, N., A. Aguas, J. D. A. van Embden, and M. T. Silva.** 1991. Mycobacteria and human autoimmune disease: direct evidence of cross-reactivity between human lactoferrin and the 65-kilodalton protein of tubercle and leprosy bacilli. Infect. Immun. **59:**1117–1125.
17. **Fischetti, V. A.** 1989. Streptococcal M protein: molecular design and biological behavior. Clin. Microbiol. Rev. **2:**286–314.
18. **Fox, A., R. R. Brown, S. K. Anderle, C. Chetty, W. J. Cromartie, H. Goodes, and J. H. Schwab.** 1982. Arthropathic properties related to the molecular weight of peptidoglycan-polysaccharide polymers of streptococcal cell walls. Infect. Immun. **35:**1003–1010.
19. **Fung, J. C., K. Wicher, and M. McCarty.** 1982. Immunochemical analysis of streptococcal group A, B, and C carbohydrates, with emphasis on group A. Infect. Immun. **37:**209–215.
20. **Gaston, J. S. H., P. F. Life, P. J. Jenner, M. J. Colston, and P. A. Bacon.** 1990. Recognition of a mycobacteria-specific epitope in the 65-kDa heat shock protein by synovial fluid-derived T cell clones. J. Exp. Med. **171:**831–841.
21. **Gillis, T. P., and T. M. Buchanan.** 1982. Production and partial characterization of monoclonal antibodies to *Mycobacterium leprae*. Infect. Immun. **37:**172–178.
22. **Hedstrom, R., J. Culpepper, R. A. Harrison, N. Agabian, and G. Newport.** 1987. A major immunogen in *Schistosoma mansoni* infections is homologous to the heat-shock protein Hsp70. J. Exp. Med. **165:**1430–1435.
23. **Holmdahl, R., R. Kristoffer, L., Klareskog, E. Larsson, and H. Wigzell.** 1986. Characterization of the antibody response in mice with type II collagen-induced arthritis using monoclonal anti-type II collagen antibodies. Arthritis Rheum. **29:**400–410.
24. **Holoshitz, I., A. Klajman, I. Druker, Z. Lapidot, A. Yaretzky, A. Frenkel, W. van Eden, and I. R. Cohen.** 1986. T lymphocytes of rheumatoid arthritis patients show augmented reactivity to a fraction of mycobacteria cross-reactive with cartilage. Lancet **ii:**305–309.
25. **Holoshitz, J., Y. Naparstek, A. Ben-Nun, and I. R. Cohen.** 1983. Lines of T lymphocytes induce or vaccinate against autoimmune arthritis. Science **219:**56–58.
26. **Jacobs, J.** 1992. Pediatric rheumatology for the practitioner. Springer-Verlag. New York.
27. **Jindal, S., A. K. Dudani, B. Singh, C. B. Harley, and R. S. Gupta.** 1989. Primary structures of a human mitochondrial protein homologous to the bacterial and plant chaperonins and to the 65-kilodalton mycobacterial antigen. Mol. Cell. Biol. **9:**2279–2283.
28. **Jones, D. B., A. F. Coulson, and G. W. Duff.** 1993. Sequence homologies between hsp60 and autoantigens. Immunol. Today **14:**115–118.
29. **Kaplan, M. H.** 1963. Immunologic relation of streptococcal and tissue antigens. J. Immunol. **90:**595–606.
30. **Karlsson-Parra, A., K. Soderstrom, M. Ferm, J. Ivanyi, R. Kiessling, and L. Klareskog.** 1990. Presence of human 65kDa heat shock protein (hsp) in inflamed joints and subcutaneous nodules of RA patients. Scand. J. Immunol. **31:**283–288.
31. **Kaufmann, S. H., B. Schoel, J. D. A. van Embden, T. Koga, A. Wand-Wurttenberger, M. E. Munk, and U. Steinhoff.** 1991. Heat-shock protein 60: implications for pathogenesis of and protection against bacterial infection. Immunol. Rev. **121:**67–90.
32. **Kelley, W. N., S. Ruddy, and C. B. Sledge.** 1989. Textbook of rheumatology. The W. B., Saunders Co., Philadelphia.
33. **Lancefield, R. C.** 1962. Current knowledge of the type-specific M antigens of group A streptococci. J. Immunol. **89:**307–313.
34. **Lehner, T., E. Lavery, R. Smith, R. van der Zee, Y. Mizushima, and T. Shinnick.** 1991. Association between the 65-kiladalton heat shock protein, *Streptococcus sanguis*, and the corresponding antibodies in Behcet's syndrome. Infect. Immun. **59:**1434–1441.
35. **Manjula, B. N., B. L. Trus, and V. A. Fischetti.** 1985. Presence of two distinct regions in the coiled-coil structure of the streptococcal PepM5 protein: relationship to mammalian coiled-coil proteins and implications to its biological properties. Proc. Natl. Acad. Sci. USA **82:**1064–1068.
36. **Morrison, R. P., R. J. Belland, K. Lyng, and H. D. Caldwell.** 1989. Chlamydial disease pathogenesis: the 57-kDa chlamydial hypersensitivity antigen is a stress response protein. J. Exp. Med. **170:**1271–1280.
37. **Mouw, A. R., E. H. Beachey, and V. Burdett.** 1988. Molecular evolution of streptococcal M protein: cloning and nucleotide sequence of type 24 M protein gene and relation to other genes of *Streptococcus pyogenes*. J. Bacteriol. **170:**676–684.
38. **Phillips, G. N., P. F. Flicker, C. Cohen, B. N. Manjula, and V. A. Fischetti.** 1981. Streptococcal M protein: alpha-helical coiled-coil structure and arrangement on the cell surface. Proc. Natl. Acad. Sci. USA **78:**4689–4693.
39. **Plikaytis, B. B., G. M. Carlone, C. Pau, and H. W. Wilkinson.** 1987. Purified 60-kilodalton *Legionella* protein antigen with *Legionella*-specific and nonspecific epitopes. J. Clin. Microbiol. **25:**2080–2084.
40. **Res, P. C. M., F. C. Breedveld, J. D. A. van Embden, C. G. Schaar, W. van Eden, I. R. Cohen, and R. R. P. de Vries.** 1988. Synovial fluid T cell reactivity against the 65kDa heat shock protein of mycobacteria in early chronic arthritis. Lancet **ii:**478–480.
41. **Shinnick, T. M.** 1987. The 65-kilodalton antigen of *Mycobacterium tubercu-*

losis. J. Bacteriol. **169:**1080–1088.
42. **Shinnick, T. M.** 1991. Heat shock proteins as antigens of bacterial and parasitic pathogens. Curr. Top. Microbiol. Immunol. **167:**145–156.
43. **Stuart, J. M., M. A. Cremer, A. S. Townes, and A. H. Kang.** 1982. Type II collagen-induced arthritis in rats: passive transfer with serum and evidence that IgG anticollagen antibodies can cause arthritis. J. Exp. Med. **155:**1–16.
44. **Stuart, J. M., and F. J. Dixon.** 1983. Serum transfer of collagen-induced arthritis in mice. J. Exp. Med. **158:**378–392.
45. **Svartman, M., E. V. Potter, T. Poon-King, and D. P. Earle.** 1975. Immunoglobulins and complement components in synovial fluid of patients with acute rheumatic fever. J. Clin. Invest. **56:**111–117.
46. **Terato, K., K. A. Hasty, R. A. Reife, M. A. Cremer, A. H. Kang, and J. M. Stuart.** 1992. Induction of arthritis with monoclonal antibodies to collagen. J. Immunol. **148:**2103–2108.
47. **Trentham, D. E., R. A. Dynesius, and J. R. David.** 1978. Passive transfer by cells of type II collagen-induced arthritis in rats. J. Clin. Invest. **62:**359–366.
48. **Trentham, D. E., A. S. Townes, and A. H. Kang.** 1977. Autoimmunity to type II collagen: an experimental model of arthritis. J. Exp. Med. **146:**857–868.
49. **van den Brock, M. F., J. M. Hogervorst, M. J. C. van Bruggen, W. van Eden, R. van der Zee, and W. B. van den Berg.** 1989. Protection against streptococcal cell wall-induced arthritis by pretreatment with the 65kDa mycobacterial heat shock protein. J. Exp. Med. **170:**446–449.
50. **van Eden, W.** 1991. Heat shock proteins and the immune system. Immunol. Rev. **121:**5–28.
51. **van Eden, W., J. Holoshitz, Z. Nevo, A. Frenkel, A. Klajman, and I. R. Cohen.** 1985. Arthritis induced by a T-lymphocyte clone that responds to *Mycobacterium tuberculosis* and to cartilage proteoglycans. Proc. Natl. Acad. Sci. USA **82:**5117–5120.
52. **van Eden, W., J. Thole, R. van der Zee, A. Noordzjiz, J. D. A. van Embden, E. J. Hensen, and I. R. Cohen.** 1988. Cloning of the mycobacterial epitope recognized by T lymphocytes in adjuvant arthritis. Nature (London) **331:** 171–173.
53. **Voller, A., D. Bidwell, and A. Bartlett.** 1980. Enzyme linked immunosorbent assay, p. 359–371. *In* N. R. Rose and H. Friedman (ed.), Manual of clinical immunology. American Society for Microbiology, Washington, D.C.
54. **Watson, K.** 1990. Microbial stress proteins. Adv. Microbiol. Physiol. **31:**183–223.
55. **World Health Organization.** 1995. Results of a World Health Organization-sponsored workshop on monoclonal antibodies to *Mycobacterium leprae*. Infect. Immun. **48:**603–605.
56. **Zhang, Z. J., C. S. Y. Lee, O. Lider, and H. L. Weiner.** 1990. Suppression of adjuvant arthritis in Lewis rats by oral administration of type II collagen. J. Immunol. **145:**2489–2493.

Editor: V. A. Fischetti