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A Protective Monoclonal Anti-Idiotypic Vaccine to Lethal Semliki Forest Virus Infection in BALB/c Mice

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Two monoclonal anti-idiotypic antibodies (ab2 MAbs), designated 1.13A112 (immunoglobulin G type 2a [IgG2a]) and 1.13A321 (IgG1), were prepared against Semliki Forest virus (SFV)-neutralizing ab1 MAb UM 1.13. They were identified in hybridoma supernatant fluid by their capacity to block UM 1.13-mediated neutralization of SFV. Although the neutralization-blocking capacities of the ab2 MAbs did not differ, only 1.13A321 evoked SFV-neutralizing ab3 antibodies upon intracutaneous and subcutaneous immunization of BALB/c mice with 1.13A321 chemically cross-linked to keyhole limpet hemocyanin and combined with the adjuvant Quil A. SFV-neutralizing ab3 antibodies appeared in serum within 10 days after primary immunization, and neutralizing antibody titers could be as high as 1/1,000 at day 35. All mice who had developed SFV-neutralizing antibodies upon anti-idiotypic immunization survived an otherwise lethal challenge with virulent SFV. However, induction of SFV-neutralizing ab3 antibodies by ab2 MAb 1.13A321 proved to be genetically restricted to BALB/c mice; even haplotype-identical ($H\text{-}2^d$) DBA/2 mice did not respond, and consequently those animals died after infection with virulent SFV.

Monoclonal anti-idiotypic antibodies (ab2 MAbs), which bear internal images of neutralization epitopes of viruses, are potentially useful as antiviral vaccines. Such ab2β MAbs evoke virus neutralizing anti-anti-idiotypic (ab3) antibodies that might protect immunized animals against virulent viral disease. Relatively few examples of neutralizing antibody-inducing ab2 MAbs are described in the literature (4, 6, 9, 11, 17, 24). Obviously, ab2 MAbs with vaccine potential are not easily identified, possibly because of deficiencies in anti-idiotypic immunization procedures and methods for screening of ab2 MAbs in hybridoma supernatant fluids. Most authors applied solid-phase enzyme immunoassay (EIA) for detection of ab2 MAbs. In that assay, deformation of ab1 MAb, including its idiotype, might occur because of binding of the MAb to plastic, which could result in a decrease of affinity of ab2 MAb to ab1 MAb. In the present study we used a solid-phase independent detection method for ab2 MAb, namely, neutralization inhibition EIA (NI-EIA). With this method, ab2 antibodies are detected by their capacity to block ab1 MAb-mediated neutralization of Semliki Forest virus (SFV) as described previously (15, 16). Moreover, the induction of ab2 serum antibodies to SFV-neutralizing ab1 MAbs was greatly facilitated by the use of the adjuvant Quil A (13). High levels of neutralization blocking ab2 antibodies were evoked upon intracutaneous and subcutaneous injection of BALB/c mice with SFV-neutralizing MAb chemically coupled to keyhole limpet hemocyanin (KLH) and combined with Quil A (15). We suppose that ab2β MAbs are neutralization-blocking antibodies, because they bind to the antigen combining site (paratope). In this study, SFV-neutralizing MAb UM 1.13 (immunoglobulin G type 2a [IgG2a]), which binds to an epitope on the E2 membrane protein of SFV, was used for anti-idiotypic immunization and detection of neutralization-blocking ab2 MAbs.

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MATERIALS AND METHODS

SFV. Both the avirulent prototype strain of SFV (5) and a virulent strain of SFV were used (2). Preparation of batches of infectious virus and general virological methods such as plaque titration have been described previously (3).

Cells and media. L cells, a continuous line of mouse fibroblasts, were maintained in Dulbecco modified Eagle's medium buffered with 0.01 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) and supplemented with 5% calf serum, 0.2% tryptose, and antibiotics.

MAbs. In the present study, MAb UM 1.13 (IgG2a) was used for anti-idiotypic immunization. MAb UM 1.13 was produced by fusion of spleen cells obtained from prototype SFV-immunized BALB/c mice and myeloma cells of line SP 2/0. The 50% plaque reduction titer of protein G (1)-Sepharose-purified MAb UM 1.13 (5 mg of protein per ml) was over 10^6 against the prototype strain of SFV as well as against the virulent strain. MAb UM 1.13 recognizes a discontinuous epitope of the E_2 glycoprotein of SFV (18).

MAb UM 5.1 (IgG2a), specific for the E_2 glycoprotein of SFV (3), was used in direct EIAs of SFV in cell culture (22).

Enzyme labeling of MAb. Horseradish peroxidase was conjugated to MAb UM 5.1 (ascitic fluid) by the periodate method (14). The freshly prepared conjugate, diluted 1:10 in phosphate-buffered saline (PBS), was stored in 1-ml portions at 4°C after addition of a crystal of thymol (Sigma) as a preservative against bacterial growth. Immediately before being used, the conjugate was diluted 1:30,000 (end dilution) in PBS plus 0.5% Tween 20.

Coupling of MAb to KLH. Protein G-purified MAb (idiotypic and anti-idiotypic) was coupled chemically to KLH (Calbiochem-Behring, La Jolla, Calif.). Purified MAb UM 1.13 (0.8 mg) in 0.2 ml of PBS was mixed with 1 mg of KLH (in 0.2 ml of distilled water), and then the two were coupled to each other by the addition of 0.06 ml of 2.5% glutaraldehyde. After 20 min of incubation at room temperature, the reaction was stopped with 0.06 ml of 0.2 M glycine. After the addition of 0.48 ml of distilled water, the mixture was

dialyzed overnight at +4°C against distilled water. Thereafter the conjugate of KLH and MAb (1.0 ml) could be used for immunization.

Mice. BALB/c ($H\text{-}2^d$), DBA/2 ($H\text{-}2^d$), and C57BL/6J ($H\text{-}2^b$) mice of either sex were obtained from the National Institute of Health and Environmental Hygiene, Bilthoven, The Netherlands. The mice were kept in the animal house of the State University of Utrecht until use at an age of 10 to 14 weeks.

Immunization of mice. Anti-idiotypic responses against KLH-conjugated MAb UM 1.13 were provoked in female BALB/c mice. Anti-anti-idiotypic antibodies were induced in BALB/c, DBA/2, and C57BL/6J mice of either sex. The conjugate of KLH and MAb (1.0 ml) was mixed with 1 ml (1 mg) of the adjuvant Quil A (13) obtained from Superfos Biosector, Vedbaek, Denmark. Each mouse was injected intracutaneously and subcutaneously with 0.1 ml of the mixture (equivalent to 40 μg of MAb and 50 μl of Quil A) at four sites (0.025 ml per site) in the neighborhood of draining lymph nodes in axillae and groins. Booster immunizations of the same antigens were administered by the same route. Blood was taken from ether-anesthetized mice by retro-orbital puncture. Individual sera were tested for the presence of antibodies.

EIA of SFV-neutralizing antibodies. The neutralizing capacity of MAb UM 1.13 was determined as described previously (22). A modified EIA was used to quantitate small amounts of SFV-neutralizing antibodies in mouse sera as follows. Serum samples were serially (twofold) diluted in Dulbecco modified Eagle's medium supplemented with 5% calf serum in wells of 96-well plates. To the serum dilutions (0.05 ml) a standard dose of 2,500 PFU of prototype SFV (0.05 ml) was added. The virus-serum mixtures were incubated for 1 h at 37°C. Subsequently, 0.01-ml aliquots of each mixture were pipetted into wells of other 96-well plates. Thereafter, 20,000 L cells (0.1 ml) were added to each well to form monolayers. Nonneutralized SFV was allowed to multiply for 18 h at 37°C, and then the L cells were fixed by the addition of 0.1 ml of 0.05% glutaraldehyde (E. Merck AG, Darmstadt, Germany) for 10 min at room temperature. After the cells were washed with tap water and rinsed with PBS, the direct EIA of SFV antigens in L cells was performed with horseradish peroxidase-labeled MAb UM 5.1 as described elsewhere (16, 22). Inhibition of virus multiplication by neutralizing antibody was calculated as a percentage of control: percent inhibition = $100 - [(A_{450}$ of serum dilution $- A_{450}$ of noninfected cell control)/(A_{450} of virus control $- A_{450}$ of cell control)] $\times$ 100. The A_{450} was measured with a Titertek Multiskan photometer (Flow Laboratories, Irvine, United Kingdom). The titer of immune serum can be arbitrarily defined as that dilution causing 50% inhibition. Because twofold serum dilutions were used in this study, the nearest reciprocal dilution is considered the serum neutralization titer.

EIA for determination of anti-idiotypic antibodies. Anti-idiotypic antibodies in both serum and hybridoma supernatant fluids that block neutralization of SFV by MAb UM 1.13 were detected by an NI-EIA as described previously (16). In short, aliquots (0.025 ml) of 1:400,000-diluted MAb UM 1.13 (ascitic fluid) were mixed with either serially diluted serum (0.025 ml) or ab2 MAb (in hybridoma supernatant fluid or purified from ascites). After 1 h of incubation at 37°C in wells of 96-well plates, 2,500 PFU of prototype SFV was added and incubated for 1 h at 37°C. Then 20,000 L cells (0.1 ml) were seeded into each well, and nonneutralized SFV was allowed to multiply for 18 h at 37°C. Subsequently, the direct EIA of SFV was performed as described above. Low absorbance values (due to insignificant virus multiplication) indicate that the neutralizing capacity of MAb UM 1.13 is unharmed. In contrast, high absorbance values (due to unrestrained virus multiplication) indicate blocking of the neutralizing capacity of MAb UM 1.13 by anti-idiotypic antibodies. Blocking by anti-idiotypic antibody could be expressed as percent inhibition at each dilution of either anti-idiotypic serum or anti-idiotypic MAb: percent inhibition = $100\% \times [(A_{450}$ of anti-idiotypic antibody $- A_{450}$ of MAb UM 1.13)/(A_{450} of virus control $- A_{450}$ of MAb UM 1.13)]. The titer of anti-idiotypic serum or anti-idiotypic MAb could be defined as that dilution causing 50% inhibition.

Protection experiments in mice. To test whether protection was induced by anti-idiotypic immunization, mice were infected intraperitoneally with 250 50% lethal doses (250 $LD_{50} \equiv 400$ PFU) of virulent SFV in 0.5 ml of PBS at pH 7.2. To quantitate protection, mice were observed for 21 days. Control mice generally died within 7 days after challenge.

RESULTS

Development of anti-idiotypic antibodies in serum after immunization with SFV-neutralizing MAb UM 1.13. Eighteen 10-week-old female BALB/c mice were immunized intracutaneously with MAb UM 1.13 coupled to KLH and mixed with the adjuvant Quil A to induce anti-idiotypic antibodies. Blood was obtained on days 7 ($n = 2$), 11 ($n = 2$), 15 ($n = 2$), 18 ($n = 2$), 21 ($n = 2$), 24 ($n = 4$), 28 ($n = 2$), and 35 ($n = 2$). The last two mice received on day 35 a booster immunization similar to the primary immunization. Blood was obtained from these mice 2, 4, 6, and 8 days later.

The titers of anti-idiotypic antibodies were determined by NI-EIA. Standard neutralizing doses of MAb UM 1.13 (1/400,000 dilution of ascitic fluid) were mixed with serial dilutions (−10 log: 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, and 4.5) of anti-idiotypic serum.

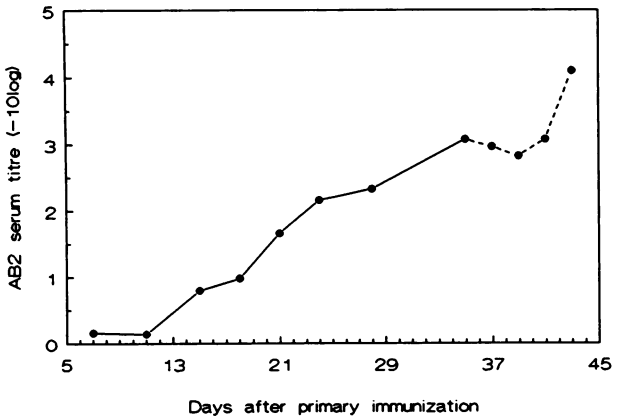

FIG. 1. Development of anti-idiotypic antibodies in serum after immunization with SFV-neutralizing MAb UM 1.13. Eighteen female BALB/c mice were immunized as described in the text. At various intervals after immunization, blood was tested for anti-idiotypic antibodies in serum that would block neutralization of SFV by MAb UM 1.13. The mean ($n = 2$ or 4) 50% inhibition [$A_{450} = 0.5(1.190 - 0.050)$] titers of anti-idiotypic sera at the indicated days after primary immunization are connected by the solid line. The mean serum titers measured in two mice at days 37, 39, 41, and 43 (after booster at day 35) are connected by the dashed line.

TABLE 1. Simultaneous development of SFV-neutralizing antibodies in serum and protection against virulent SFV after primary immunization of female BALB/c mice with anti-idiotypic MAb 1.13A321 and adjuvant Quil A[a]

Anti-idiotypic MAb used as vaccine[b]	Interval (days) between immunization and challenge	Reciprocal titers of SFV-neutralizing ab3 antibodies in serum[c]	No. of surviving mice/no. of infected[d] mice
1.13A321	7	<4, <4, <4, <4, 8, 8	2/6
1.13A321	21	<4, 16, 16, 32, 32, 64	5/6
1.13A321	35	256, 256, 512, 512, 512, 1,024	6/6
21.1A1[e]	10	<5, <5, <5, <5, <5	0/5
1.13A321	10	<5, 5, 5, 20, 20	5/5

[a] Results of two separate experiments.
[b] Mice were intracutaneously and subcutaneously immunized with KLH-coupled ab2 (40 μg per animal) and Quil A (50 μg per animal).
[c] Obtained from individual mice just before challenge.
[d] Mice were intraperitoneally injected with 400 PFU (250 LD_{50}) of virulent SFV in 0.5 ml of PBS at pH 7.2. Untreated control mice (n = 5 or 6) all died.
[e] An ab2 MAb (IgG2a) specific for encephalomyocarditis virus-neutralizing MAb UM 21.1 (23).

As shown in Fig. 1, female BALB/c mice developed neutralization-blocking antibodies after a single immunization. The presence of anti-idiotypic antibodies became obvious at day 15. Thereafter the antibody level of ab2 rose steadily until day 35 in this experiment. After booster immunization at day 35, the steep rise in antibody titer occurred rather late (between day 6 and 8), and therefore fusions of immune spleen cells and myeloma cells were performed 5 days after booster injection.

Production of anti-idiotypic MAbs to MAb UM 1.13. In two fusion experiments, blocking ab2 antibodies in hybridoma supernatant fluid were detected by NI-EIA. Subcloned hybridomas were injected into pristane-primed mice. The ab2 MAbs in the resulting ascitic fluid were purified by protein G-Sepharose column chromatography. Two excellently blocking ab2 MAbs were obtained: MAb 1.13A112 (IgG2a) and MAb 1.13A321 (IgG1). The standard neutralizing dose of MAb UM 1.13 (0.025 ml; ascitic fluid, 1/400,000) was blocked significantly by 7.5 ng (in 0.025 ml) of either purified ab2 MAb. Blocking did not occur with 2.5 ng of ab2 MAb (results not shown).

Simultaneous induction of neutralizing antibodies and protection by anti-idiotypic immunization. MAb 1.13A321 was able to induce SFV-neutralizing antibodies in BALB/c mice (Tables 1 and 2). The rapid induction of both neutralizing antibodies and protection against virulent SFV is exemplified by the following experiment. Separate groups (n = 6) of female BALB/c mice were immunized intracutaneously and subcutaneously with KLH-coupled ab2 MAb 1.13A321 and the adjuvant Quil A at fortnightly intervals (−5, −3, and −1 week) before intraperitoneal challenge with 250 LD_{50} of the virulent strain of SFV. Just before challenge, blood was obtained from all mice to quantitate SFV-neutralizing antibodies in serum. Serum antibodies were titrated by N-EIA using twofold (4 to 1,024) dilutions of serum. The results are presented in Table 1. Seven days after immunization, two of six mice had developed neutralizing antibodies which were protective, as indicated by the survival of these mice after an otherwise lethal challenge. By day 21, most mice (five of six) had neutralizing antibodies, and these mice were also protected. By day 35, all mice had developed high levels of protective neutralizing antibodies. Untreated control mice all died within 7 days after challenge.

In a second experiment, groups of mice were immunized with ab2 MAb 1.13A321 and ab2 MAb 21.1A1 (specific for encephalomyocarditis virus-neutralizing MAb UM 21.1 [23]; our unpublished results). These mice were challenged on day 10 with 250 LD_{50} of SFV. As shown in Table 1, mock-immunized mice all died, but mice immunized with ab2 MAb 1.13A321 all survived. Four of the five mice had detectable SFV-neutralizing antibodies before challenge. In the two experiments described above, surviving mice remained without any signs of SFV infection such as ruffled fur or paralytic hind legs.

Genetic restriction of protection by ab2 MAb. In the next experiment, other strains of mice were immunized with ab2 MAb 1.13A321 to establish whether successful vaccination was restricted to BALB/c mice. As a control, ab2 MAb 1.13A112 was used for mock immunization.

Groups (n = 6) of BALB/c ($H\text{-}2^d$), DBA/2 ($H\text{-}2^d$), and C57BL/6J ($H\text{-}2^b$) mice of either sex were immunized intracutaneously and subcutaneously with ab2 MAbs coupled to KLH and mixed with the adjuvant Quil A. The mice were given boosters on day 33. Control mice of the same age were not treated. Blood was taken from ether-anesthetized mice 7 days before challenge at day 48 by retro-orbital puncture. The individual sera were assayed for neutralizing antibody content. At day 55, all mice were challenged intraperitoneally with 400 PFU (250 LD_{50}) of the pathogenic strain of SFV and were monitored for protection for 21 days. Nonsurviving mice all died within 8 days after challenge. However, as shown in Table 2, induction of neutralizing ab3 antibodies proved to be genetically restricted to BALB/c mice. DBA/2 and C57BL/6J mice developed no neutralizing ab3 antibodies, and all immunized mice died after intraperitoneal challenge with virulent SFV.

TABLE 2. Genetic restriction of anti-idiotypic protection against lethal SFV to BALB/c mice

Mouse strain	Sex	ab2 MAb used for immunization[a]	SFV-neutralizing antibody titers (ab3)[b]	No. of survivors/no. infected
BALB/c	Female	1.13A112	ND	1/6
BALB/c	Female	1.13A321	5, 10, 10, 40, 40, 80	6/6
BALB/c	Male	1.13A112	ND	0/6
BALB/c	Male	1.13A321	ND, 10, 10, 40, 160, 160	5/6
DBA/2	Female	1.13A321	ND	0/6
DBA/2	Male	1.13A321	ND	0/6
C57BL/6J	Female	1.13A321	ND	0/6

[a] Mice were intracutaneously and subcutaneously immunized with KLH-coupled ab2 MAb (40 μg per animal) and adjuvant Quil A (50 μg per animal). The mice were given similar boosters at day 33 of immunization. Mice were infected intraperitoneally at day 55 of immunization with 250 LD_{50} of SFV in 0.5 ml of PBS at pH 7.2.
[b] Determined by N-EIA using twofold dilutions (5 to 1,280) of serum. Titers were for sera from individual mice 7 days before challenge. ND, Not detected.

DISCUSSION

In this article we describe a monoclonal ab2 vaccine which protected BALB/c mice against an otherwise lethal infection with SFV. The observed protection was due to induction of SFV-neutralizing antibodies by anti-idiotypic immunization with MAb UM 1.13A321. As shown in Table 1, neutralizing antibodies appear rapidly in serum within 10 days after primary intracutaneous and subcutaneous immunization with KLH coupled to 1.13A321 and the adjuvant Quil A. Protection appeared concomitantly. Mock-immunized BALB/c mice all died, indicating that nonspecific immunity, which could possibly have been induced by the immunization procedure alone, was not involved.

After primary immunization with MAb UM 1.13A321, the level of SFV-neutralizing antibodies rose steadily until at least day 35, reaching titers over 1/100 or even over 1/1,000 after booster immunization. Such neutralization titers are comparable to those measured after infection of BALB/c mice with an avirulent strain of SFV (12).

In contrast to ab2 MAb UM 1.13A321 (IgG1), another ab2 MAb UM 1.13A112 (IgG2a) was unable to induce either neutralizing antibodies or protection (Table 2). However, both ab2 MAbs blocked the neutralizing capacity of MAb UM 1.13 equally well. This result indicates that neutralization (antigen)-blocking ab2 MAbs are not per se antibodies bearing the internal image of the antigen.

Both ab2 MAbs were recognized in hybridoma supernatant fluid by their neutralization-blocking capacity in the NI-EIA. Beforehand, we assumed that neutralization-blocking ab2 antibodies would be, foremost, antibodies binding to the paratope (the actual antigen-binding site) of virus-neutralizing ab1 MAbs. Among paratope-blocking antibodies (7, 8, 10) would be the ab2 MAbs looked for, i.e., those that bear the internal image of the antigen and that could be used as surrogate vaccine (4, 6, 8, 17, 20, 21). Conventional solid-phase EIA may be less suitable for detecting the right ab2 MAbs. In solid-phase EIA, the paratope of ab1 might be conformationally changed by coating it with plastic, thus causing diminished sensitivity of the detection system.

A prerequisite for the detection of ab2 MAbs is adequate immunization of mice whose spleens are destined for fusion. In agreement with earlier reports (15, 16), we demonstrate in the present study that an immunization protocol with KLH-coupled ab1 MAb and Quil A is very useful for induction of ab2 antibodies (Fig. 1). Conspicuous antibody formation after booster immunization at day 35 is rather late, and therefore fusions were not performed on day 3, but they were advantageously performed on day 5 after the booster.

As shown in Table 2, induction of SFV-neutralizing antibodies and thereby protection against lethal infection is restricted to BALB/c ($H\text{-}2^d$) mice. C57BL/6J ($H\text{-}2^b$) and even haplotype-identical ($H\text{-}2^d$) DBA/2 mice developed no neutralizing antibodies upon injection of MAb UM 1.13A321 and Quil A, and consequently all those mice died after intraperitoneal challenge with virulent SFV. Nevertheless, we expect that SFV infection in mice will prove to be an excellent model for further development and refinement of monoclonal anti-idiotypic vaccines. In recent experiments we successfully used recombinant DNA protein fragments of SFV (19) instead of KLH as carrier molecules. Currently we are investigating whether these protein fragments, which contain T-cell epitopes of SFV, can provide adequate antigen-specific T-cell memory.

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