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Immune Responses in Mice Against Herpes Simplex Virus: Mechanisms of Protection Against Facial and Ganglionic Infections

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We performed experiments with mice to determine the nature of the immune response(s) that prevents primary infections of the skin and the trigeminal ganglia with herpes simplex virus. Immunization with infectious herpes simplex virus, inactivated virus, or material enriched for viral glycoproteins protected hairless mice against primary facial and ganglionic infections. Live and inactivated viruses induced neutralizing antibodies, whereas glycoprotein material did not. Instead, glycoprotein material induced antibodies that were largely directed against two glycopolypeptides with molecular weights of 120,000 to 130,000. Hairless mice immunized with glycoprotein material responded faster than control mice in the synthesis of neutralizing antibodies after challenge with infectious virus. Congenital athymic BALB/c (nu/nu) mice were protected against primary facial infections after immunization with glycoprotein material, but glycoprotein-specific antibodies were not induced.

Exposure of humans or experimental animals to a live virus generally induces long-lasting immunity and protection against reinfection with the same or a closely related virus. Herpes simplex virus (HSV) is an exception; reactivation of latent virus in nerve tissues causes recurrent infections (1, 20, 42) despite active humoral and cellular immunities (7, 8, 24), and primary genital infections with HSV type 2 (HSV-2) can occur in individuals that already have developed immunity against the serologically related virus HSV-1 (31, 39). More detailed knowledge of the immune responses during primary and recurrent herpetic infections is needed to understand this paradox. Furthermore, such knowledge would be helpful in developing and evaluating herpesvirus vaccines. A safe vaccine has to contain viral antigens without nucleic acids because of the oncogenic potential of herpesvirus and its deoxyribonucleic acid (9, 12, 15, 36, 46).

The purpose of this work was to compare three different viral preparations for their efficiency in protecting hairless mice against primary ganglionic infections with HSV-1 and for their ability to generate a number of different virus-specific immune responses. By correlating these two phenomena, we hoped to gain an understanding of the important immune parameters in protection against herpetic infections.

The viral preparations used were infectious virus, purified inactivated virus, and a fraction enriched for viral glycoproteins. Each of these preparations should generate a different spectrum of immune responses, with the responses against infectious virus being the most extensive and varied and the responses against glycoprotein material being the most restricted.

MATERIALS AND METHODS

Buffers and solutions. NET buffer contained 0.05 M tris(hydroxymethyl)aminomethane, 0.005 M ethylenediaminetetraacetate, 0.15 M NaCl, and 0.1 mM phenylmethylsulfonyl fluoride (pH 7.2). NET-T buffer was NET buffer containing 0.05% Triton X-100. Phosphate-buffered saline (PBS) contained 0.04 M sodium phosphate and 0.15 M NaCl (pH 7.2); PBS-KCl was PBS plus 0.005 M KCl. SP buffer contained 0.01 M tris(hydroxymethyl)aminomethane (pH 6.8), 2% sodium dodecyl sulfate (SDS), 2% β-mercaptoethanol, 20% glycerol, and 0.02% bromophenol blue.

Cells and virus. Primary rabbit kidney (PRK) cells were prepared from 6-week-old New Zealand white rabbits and were grown in Eagle minimal essential medium (EMEM) containing 10% fetal calf serum. Vero cells were grown in the same medium.

The Tyler strain of HSV-1 was obtained from A. Friedman, Merck Institute for Therapeutic Research. This virus was plaque-purified three times in PRK cells under carboxymethyl cellulose (35), and the viral yield from one plaque was used to infect 10^6 PRK cells at a multiplicity of infection of 0.01 plaque-forming unit per cell in EMEM without fetal calf serum. Virus was harvested 36 to 48 h later, when extensive cytopathology appeared in the culture, and this virus was used to prepare a working stock by infecting 5×10^7 PRK cells at a multiplicity of infection of 0.01.

Virus was purified as described previously (23) from the supernatant of infected PRK cells which were grown in roller bottles and inactivated with ultraviolet

light (2.5×10^6 ergs/cm^2) and heat (55°C for 30 min). After this treatment, infectious virus could not be detected after this material was passaged three times in PRK cells.

Mice and virus challenge. Hairless HRS/J mice (21) were obtained from R. J. Klein, New York University School of Medicine, and were maintained as a closed colony by Buckshire Farms, Perkasie, Pa. When they were 2 to 3 months old, they were scratched superficially on the forehead and the snout with a 26-gauge needle, and 0.05 ml of HSV-1 Tyler (2×10^6 to 4×10^6 plaque-forming units per ml) was spread over the scratched skin. Severe facial lesions developed after 5 to 7 days but disappeared after 14 days.

Congenital athymic (nu/nu) and control (nu/+) BALB/c mice were obtained from AR Sprague Dawley, Madison, Wis. nu/nu mice were infected as described above for hairless mice; nu/+ mice were infected after the lip area was scratched.

Latent HSV in trigeminal ganglia. Trigeminal ganglia were excised 4 to 6 weeks after mice were challenged, and these ganglia were incubated at 37°C in 1 ml of EMEM containing 2% fetal calf serum for 3 days. Then they were sonicated, the debris was removed by centrifugation at 1,000 rpm for 10 min, and 0.2 ml of the supernatant was added to MRC-5 embryonic lung cells (43; P. Morahan, personal communication). Cultures were kept for 3 weeks and scored for herpesvirus-specific cytopathic effects, which generally appeared in positive cultures after 5 or 6 days.

Preparation of glycoprotein material. Infected PRK cells from 10 roller bottles (area, 850 cm^2 each) were scraped into PBS, washed once with PBS, and lysed in ml of NET buffer containing 1% Triton X-100 (15 min at 4°C). Nuclei were removed by centrifugation at 1,500 rpm for 5 min and macromolecular structures were removed by centrifugation at $100,000 \times g$ for 120 min. The supernatant was irradiated with ultraviolet light (2.5×10^6 ergs/cm^2) and applied to a 10-ml (wet volume) column containing the lectin from *Lens culinaris* coupled to Sepharose 4B. The column was washed with NET-T buffer, and adsorbed material was eluted with the same buffer containing 10 mg/ml of α-methyl-mannoside and glucose. A sample of this material was mixed with SDS (final concentration, 0.1%) and precipitated with 4 volumes of acetone. Pelleted material was washed with acetone and used for protein determination.

Immunization. Mice were immunized with infectious virus by injecting 0.05 ml of HSV-1 Tyler (2×10^5 plaque-forming units per ml) into a footpad. Inactivated virus and glycoprotein material were adsorbed to aluminum hydroxide by slowly adding 0.85 ml of $AlK(SO_4)_2 \cdot 12H_2O$ per 10 ml of a solution containing 0.32 mg of protein per ml. The pH was maintained at 6 by adding NaOH. The precipitates were stirred for 2 h, pelleted by centrifugation at $1,300 \times g$ for 25 min, and suspended in 0.9% NaCl so that the suspensions contained 100 μg of protein per ml for purified virus and 200 μg of protein per ml for glycoprotein material. Mice were immunized by injecting 0.1 ml of alum-adsorbed material intraperitoneally.

Sera. Mouse sera were obtained by bleeding from the inferior vena cava. Rabbit serum against HSV-1 Tyler was prepared in 2-month-old New Zealand white rabbits. PRK cells were infected at a multiplicity of infection of 0.01 in EMEM without fetal calf serum, and they were washed with PBS when extensive cytopathic effects appeared. Cells were scraped into PBS, and a 10% (vol/vol) cell suspension was prepared; this suspension was stored at −70°C after sonication. A 0.25% cell suspension was mixed with an equal volume of complete Freund adjuvant and injected intradermally on the backs of rabbits at five locations (0.2 ml each), and 1.5 ml of the 0.25% cell suspension was injected intraperitoneally. After 3 weeks, the rabbits were boosted by injecting 2 ml of a 2.5% infected cell suspension intraperitoneally, and 10 days later rabbits were bled by heart puncture. Control sera were prepared similarly with uninfected PRK cells. All sera were heated for 30 min at 56°C.

Neutralizing antibody determinations. Complement-dependent neutralizing antibody titrations were performed in quadruplicate in 96-well Microtest II tissue culture plates (Falcon Plastics). Each well received 50 μl of serially diluted serum, 125 plaque-forming units of HSV-1 Tyler in 25 μl, and 25 μl of rabbit complement diluted 1:10 (all in EMEM). Plates were incubated for 3 h at 37°C, and this was followed by the addition of 10^4 Vero cells in 50 μl of EMEM containing 6% heat-inactivated fetal calf serum. Wells were read for cytopathic effects after 3 days at 37°C, and neutralizing titers were expressed as the inverse of the highest serum dilution that prevented cytopathic effects in at least two of four wells.

Radiolabeling of infected cells. Vero or PRK cells (in 75-cm^2 Falcon flasks) were infected at a multiplicity of infection of 0.1 in EMEM without fetal calf serum. At 16 h postinfection, the cells were washed with PBS and incubated with 5 ml of EMEM lacking methionine. After 30 min 100 μCi of [^{35}S]methionine (500 Ci/mmol; Amersham/Searle) was added. After 4 h the cells were washed twice with complete EMEM, incubated for 1 h in EMEM, and removed from the surface of the flask with 0.02% ethylenediaminetetraacetate–0.05% $NaHCO_3$ in PBS-KCl. They were washed once in PBS and stored at −70°C as pellets containing 10^6 cells. Unlabeled uninfected Vero or PRK cells were harvested similarly and stored as pellets containing 3×10^6 cells.

Immune precipitation. Protein A-bearing *Staphylococcus* (Staph A) immunoadsorbent (IgGsorb) was purchased from Enzyme Center, Inc., Boston, Mass. It was reconstituted to yield a 10% (vol/vol) suspension and was kept at −70°C in 2-ml portions. Before use the bacteria were washed as described previously (17) and suspended in NET-T buffer.

Each frozen cell pellet (1×10^6 ^{35}S-labeled infected cells or 3×10^6 unlabeled uninfected cells) was suspended in 200 μl of NET buffer. Triton X-100 and deoxycholate were each added to 1%, and the suspension was kept at 4°C for 15 min. Nuclei and large particulate material were removed by centrifugation at $700 \times g$ for 6 min, and the supernatant was preadsorbed for 60 min at 22°C with 20 μl of normal rabbit serum; this was followed by the addition of 100 μl of Staph A (20%, vol/vol) for 15 min at 22°C. This mixture was centrifuged at $6,500 \times g$ for 2 min, and the pellet was discarded. Samples (20 μl) of diluted serum (generally diluted 1:25) were preadsorbed for 15

min at 22°C with 20 μl of extract prepared from homologous uninfected, unlabeled cells. To this 20 μl of preadsorbed labeled extract was added, and this mixture was incubated for 60 min at 22°C; this was followed by the addition of 50 μl of Staph A suspension (10%, vol/vol). After incubation for 15 min at 22°C, complexes were pelleted by centrifugation at 6,500 × *g* for 2 min, and the pellets were washed four times with 1.0 ml of NET-T buffer. The final pellet was suspended in 70 μl of SP buffer and placed in boiling water for 3 min to dissociate the antigen-antibody complexes, and this was followed by the removal of Staph A by centrifugation and analysis of the supernatant by SDS-polyacrylamide gel electrophoresis.

SDS-polyacrylamide gel electrophoresis. Samples were electrophoresed in 10% discontinuous slab gels for 5 h at 110 V (25). Gels were fixed for 30 min in a mixture containing methanol, acetic acid, and water (5:1:5), and then they were soaked overnight in 7% acetic acid. Next, the gels were impregnated with 2,5-diphenyloxazole (22.4 g/100 ml of dimethyl sulfoxide), rinsed with water, dried, and exposed to Kodak Royal X-Omat film (22). Molecular weight markers (Pharmacia Fine Chemicals) were phosphorylase *b* (molecular weight, 94,000), albumin (67,000), ovalbumin (43,000), carbonic anydrase (30,000), and trypsin inhibitor (20,100). These markers were labeled with [^{14}C]-acetic anhydride by suspending the contents of a Pharmacia vial in 1 ml of 0.3 M phosphate buffer (pH 7.2) and adding 500 μCi of [^{14}C]acetic anhydride (60 to 120 mCi/mmol; Amersham/Searle). This mixture was incubated for 30 min at room temperature, and labeled proteins were lyophilized after purification by chromatography on a P-10 column (Pharmacia Fine Chemicals). The molecular weights of the glycoproteins were estimated in relation to the reovirus polypeptides with molecular weights of 155,000, 140,000, and 72,000 (40).

Radioimmunoprecipitation of viral glycoproteins. A total of 2 × 10^6 [^{35}S]methionine-labeled Vero cells infected with HSV-1 Tyler were lysed with 1% Triton X-100 in 0.5 ml of NET buffer. Nuclei were removed, and the cytoplasmic extract was fractionated by lectin affinity chromatography (see above), using 0.5-ml (wet volume) columns. The glycoprotein material was dialyzed against NET-T buffer and diluted in the same buffer to contain 2,000 cpm/20 μl. Serum dilutions were made in NET buffer, and 20 μl was added to 20 μl of the glycoprotein material. This mixture was incubated at room temperature for 2 h; 50 μl of Staph A (10%, vol/vol) was added, and the incubation was continued for another 15 min; this was followed by centrifugation at 6,500 × *g* for 2 min and one wash of the pellet in NET-T buffer. The radioactivity in the combined supernatants was determined by adding them to 10 ml of Aquasol (New England Nuclear Corp., Boston, Mass.). Pelleted material was suspended in 0.2 ml of SP buffer without bromophenol blue and was then added to 10 ml of Aquasol. Percent precipitation was determined as follows: [(counts per minute in pellet)/(counts per minute in pellet + counts per minute in supernatant)] × 100.

RESULTS

Protection against primary facial and ganglionic infections. Three preparations were tested for their ability to protect hairless mice against primary facial and ganglionic infections. These were infectious virus, inactivated purified virions, and glycoprotein material. The last was prepared by lectin affinity chromatography of cytoplasmic extracts from PRK cells infected with HSV-1 Tyler. The nature of the viral antigens in this material was evaluated as follows. [^{35}S]methionine-labeled cytoplasmic extracts from HSV-1 Tyler-infected PRK cells were reacted before and after affinity chromatography with control and immune rabbit sera, and precipitated antigens were identified by SDS-polyacrylamide gel electrophoresis (Fig. 1). Whereas a large number of viral antigens were precipitated from unfractionated cytoplasmic extract, only one major antigen was precipitated from the glycoprotein material. Previously, we showed that this antigen is a viral glycoprotein which has a molecular weight of 120,000 to 130,000 and is expressed at the surfaces of infected cells (it incorporated [^{3}H]glucosamine and was labeled with ^{125}I when infected cells were iodinated in the presence of lactoperoxi-

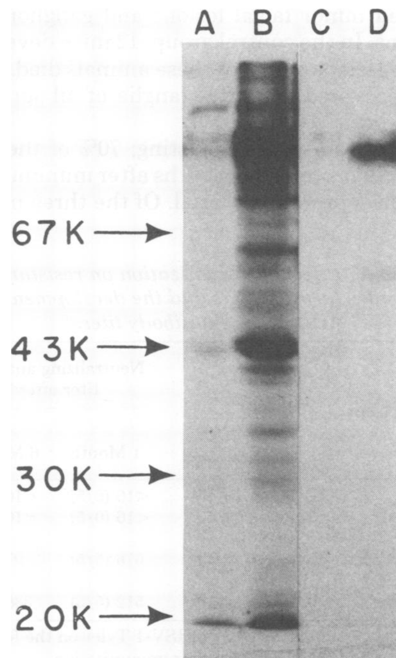

FIG. 1. *Fluorogram of polypeptides precipitated from extracts of PRK cells infected with HSV-1 Tyler and labeled with [^{35}S]methionine with control serum and rabbit serum against HSV-1 Tyler. Lane A, Cytoplasmic extract plus control serum; lane B, cytoplasmic extract plus immune serum; lane C, glycoprotein material prepared from the cytoplasmic extract by lectin affinity chromatography plus control serum; lane D, glycoprotein material plus immune serum.*

dase [Zweerink and Stanton, submitted for publication]). It corresponds in all likelihood to the A and B glycoproteins described by Spear (41). It is believed that these antigens play an important role in the recognition and lysis of HSV-infected cells (11).

Mice were immunized with live virus in a footpad. This establishes a latent infection in the lumbo-sacral ganglia and protects mice against infection of the trigeminal ganglia when they are challenged on the forehead and snout (21, 34, 38). Alum-adsorbed inactivated virions and glycoprotein material were injected intraperitoneally.

Table 1 shows that all three preparations protected hairless mice against facial lesions and ganglionic infections. When challenged 1 month after immunization, none of the mice immunized with live virus developed facial lesions, and infectious virus could not be recovered from the trigeminal ganglia; 2 of 10 mice immunized with inactivated virus developed minor facial lesions, and infectious virus was recovered from the trigeminal ganglia of these 2 mice; and 3 of 10 mice immunized with the glycoprotein material developed minor facial lesions and ganglionic infections. In the control group, 12 mice developed severe lesions, and 4 of these animals died. Virus was recovered from the ganglia of all surviving lesion-positive mice.

Protection was long lasting; 70% of the mice were still protected 6 months after immunization with glycoprotein material. Of the three mice in this group that developed facial lesions and trigeminal ganglionic infections, two had severe lesions and one had minor lesions.

TABLE 1. *Effect of immunization on resistance to primary facial lesions and the development of neutralizing antibody titers*[a]

Immunogen	No. of mice with facial lesions[b]/total no. after:		Neutralizing antibody titer after:[c]	
	1 Month	6 Months	1 Month	6 Months
Saline	12/14[d]	12/15	<16 (0/5)[e]	<16 (0/5)
Glycoprotein[f]	3/10[g]	3/10[h]	<16 (0/5)	<16 (0/5)
Inactivated virus[f]	2/10[g]	5/10[g]	676 (5/5)	97 (5/5)
Live virus[i]	0/10	1/10[g]	512 (5/5)	338 (5/5)

[a] Mice were challenged with HSV-1 Tyler on the forehead and snout at 1 and 6 months after immunization.

[b] Infectious virus was recovered from ganglia of mice with lesions; none was recovered from mice without lesions.

[c] Expressed as geometric mean titers.

[d] Four animals with lesions died.

[e] Numbers in parentheses are number of mice that seroconverted (titer, ≥16)/number tested.

[f] One dose of alum-adsorbed material given interperitoneally.

[g] Lesions were less severe than in saline-treated mice.

[h] Two mice had severe lesions (like saline-treated mice).

[i] HSV-1 Tyler was injected into a footpad.

Neutralizing antibody titers after immunization. Neutralizing antibody titers were determined in the sera of five mice in each of the three immunized groups before challenge. Mice immunized with live virus and mice immunized with inactivated virus had high neutralizing antibody titers after 1 month (titers of 512 and 676, respectively). After 6 months these titers were reduced somewhat for the group immunized with live virus (titer decreased from 512 to 335) and considerably for the group immunized with inactivated virions (titer decreased from 676 to 97). Glycoprotein material did not induce neutralizing antibodies in any of the immunized mice.

Protection by glycoprotein material without concomitant induction of neutralizing antibodies suggests a number of possible mechanisms of immune resistance. (i) Immunization with glycoprotein material may prime neutralizing antibody-producing cells such that after challenge a more rapid response occurs and thus prevents the spread of virus. (ii) Glycoprotein material may protect by inducing antibodies that do not neutralize virions in vitro. And (iii) glycoprotein-induced protection may not be antibody mediated, but may be mediated through other parameters, such as cell-mediated immunity. These possibilities were investigated further.

Kinetics of the production of neutralizing antibodies after challenge. A group of hairless mice was immunized with the glycoprotein material, and 1 month later these animals (and animals in a control group) were challenged on the forehead with HSV-1 Tyler. Neutralizing antibody titers were determined for serum samples taken at varying times after challenge.

Table 2 shows that neutralizing antibodies developed in the immunized group of mice slightly faster than in the control group. Immunized mice developed significant levels (geometric mean titer, 32) within 5 days, whereas the same levels developed in 6 days in the control group. After 7 days neutralizing antibody titers were intermediate in both groups (titers of 203 and 323 for immunized and control mice, respectively), and after 8 days titers were high in both groups.

Development of antibodies specific for the glycoprotein material after immunization or virus challenge or both. A preparation of [^{35}S]methionine-labeled glycoproteins was used in radioimmunoprecipitation assays to quantitate glycoprotein-specific antibodies in the serum samples shown in Table 2. These antibodies were not present in control mice be-

fore challenge (Fig. 2); low levels were present on day 7 after challenge, and high levels were present on day 15. High levels of glycoprotein-specific antibodies were present 1 month after immunization with the glycoprotein material, and these levels did not change significantly after challenge. High levels of glycoprotein-specific antibodies were still present 6 months after immunization (data not shown).

Specificity of antibodies induced with glycoprotein material. The specificity of the antibodies induced with glycoprotein material

TABLE 2. *Induction of neutralizing antibodies after virus challenge in glycoprotein-immunized and control mice*

Days after challenge[a]	Neutralizing antibody titers[b]	
	Immunized mice[c]	Control mice[d]
−2	<16, <16	<16, <16
2	<16, <16	<16, <16
4	<16, <16, <16	<16, <16, <16
5	16, 32, 32, 64 (32)	<16, <16, <16, 64 (2.8)
6	64, 128, 256, 256 (152)	16, 16, 32, 128 (32)
7	64, 256, 512 (203)	64, 512, 1,024 (323)
8	1,024, 1,024, 2,048 (1,290)	1,024, 1,024, 1,024 (1,024)
15	512, 2,048 (1,024)	2,048, 2,048 (2,048)

[a] Mice were challenged with HSV-1 Tyler on the snout 1 month after immunization.
[b] Neutralizing antibodies were determined in individual mice. Numbers in parentheses are geometric mean titers (a value of 1 was used if the titer was <16).
[c] Mice were immunized with glycoprotein material from PRK cells infected with HSV-1 Tyler; 4 of 16 immunized mice developed lesions.
[d] Of 10 control mice, 8 developed lesions.

was determined by reacting sera taken from mice 4 weeks after immunization with [^{35}S]methionine-labeled total cytoplasmic or lectin-purified extracts from PRK cells infected with HSV-1 Tyler. Sera from nonimmunized mice were included, as well as sera from immunized and nonimmunized mice after virus challenge. Figure 3 shows representative samples. Sera from immunized mice (before challenge) reacted mostly with the 120,000- to 130,000-dalton glycopolypeptides. After challenge nonimmunized mice developed antibodies that reacted with a large number of viral antigens (Fig. 3, lane D), whereas immunized and challenged mice developed fewer antibodies (Fig. 3, lane C). Antibodies that would not bind to Staph A (3) were not present in these sera, since the addition of rabbit antimouse immunoglobulin M or immunoglobulin G to the reaction mixture before the addition of Staph A did not alter the immunoprecipitation pattern (data not shown).

Immunization with glycoprotein material and thymus-dependent protection against primary infections. The results discussed above showed a correlation between the presence of antibodies against herpesvirus glycoproteins and protection against facial and ganglionic infections. Thus, protection could be mediated through glycoprotein-specific antibodies. However, other mechanisms of protection cannot be excluded.

The role of T-cell-mediated immunity in protection after immunization with glycoprotein material was investigated by using congenital athymic nu/nu and control nu/+ BALB/c mice. Table 3 shows that both nu/nu and nu/+ mice

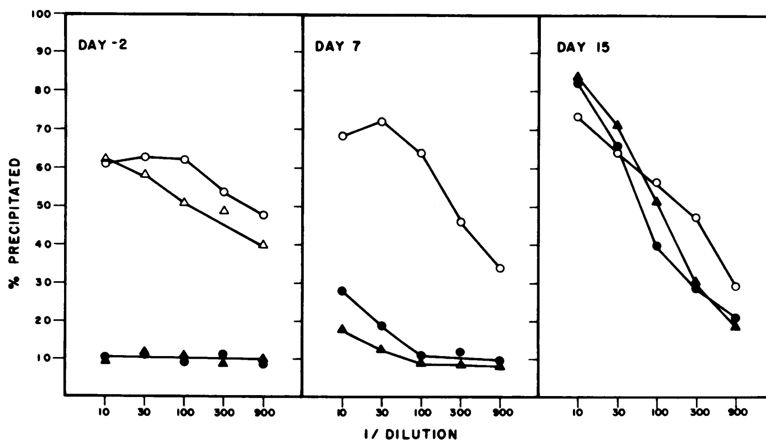

FIG. 2. *Titration of glycoprotein-specific antibodies in mouse sera. Hairless mice were immunized with glycoprotein material, and 1 month later they were challenged with HSV-1 Tyler. A nonimmunized group was challenged at the same time. Sera were taken 2 days before challenge and 7 and 15 days after challenge, and a number of individual samples were titrated for their reactivity with [^{35}S]methionine-labeled glycoprotein material. Symbols: ● and ▲, sera from nonimmunized mice; ○ and △, sera from immunized mice.*

were protected against the development of facial lesions after immunization with the glycoprotein material. The lesions that did develop on immunized mice were less severe than those that developed on control mice. However, 100% of the nu/nu mice with lesions (including minor ones) died between 2 and 6 weeks after challenge. None of the nu/nu mice without lesions died, and none of the nu/+ mice died, regardless of whether they developed lesions.

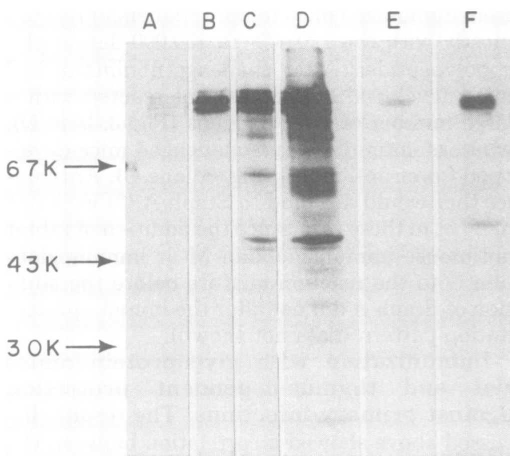

FIG. 3. *Specificity of antibodies induced with glycoprotein material. PRK cells infected with HSV-1 Tyler were labeled with [^{35}S]methionine, and a cytoplasmic extract and glycoprotein material were prepared. These fractions were reacted with different mouse sera, and precipitated antigens were analyzed by SDS-polyacrylamide gel electrophoresis. Lane A, Cytoplasmic extract plus control serum; lane B, cytoplasmic extract plus serum from immunized mouse; lane C, cytoplasmic extract plus serum from immunized mouse 3 weeks after challenge with infectious virus; lane D, cytoplasmic extract plus serum from nonimmunized and challenged mouse; lanes E and F, glycoprotein material plus sera from control and immunized mice, respectively.*

TABLE 3. *Effect of immunization on resistance to primary skin lesions and the development of neutralizing antibody titers in BALB/c (nu/nu) and (nu/+) mice*

Mice	Immunization	No. of mice with facial lesions after challenge/ total no.[a]	Neutralizing antibody titer	
			Before challenge[b]	After challenge[b]
nu/nu	Glycoprotein	3/12	<16 (0/3)[c]	<16 (0/7)
	Control[d]	13/15	<16 (0/1)	<16 (1/11)
nu/+	Glycoprotein	3/9	<16 (0/3)	69 (9/9)
	Control	12/15	<16 (0/1)	832 (10/10)

[a] Mice were challenged 4 weeks after immunization with HSV-1 Tyler.
[b] Neutralizing titers were determined 2 days before and 3 weeks after challenge.
[c] Numbers in parentheses are number of seropositive mice/ total number tested.
[d] Injected with saline.

Neutralizing antibodies developed in nu/+ mice after challenge of both the immunized group and the nonimmunized group (Table 3). Antibodies against the glycoprotein material were detected in the immunized group before challenge, and they developed to high levels at 3 weeks after challenge of the nonimmunized group (Fig. 4). Neutralizing antibodies and glycoprotein-specific antibodies were not detected in the nu/nu mice, regardless of whether they had been immunized or challenged with live virus (Table 3 and Fig. 4).

It should be noted that glycoprotein-specific antibodies were measured in four individual mice of each group. We show the results for a single mouse because the other mice in each experimental group gave identical results.

Glycoprotein-specific antibodies that would not bind to Staph A (3) were also absent in the sera of nu/nu mice. This was established by

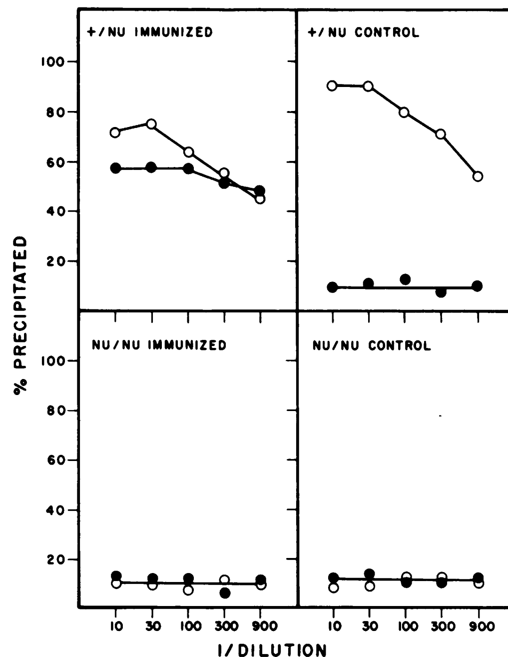

FIG. 4. *Titration of glycoprotein-specific antibodies in nu/nu and nu/+ BALB/c mice. Mice were immunized with glycoprotein material, and 4 weeks later they were challenged with HSV-1 Tyler. Nonimmunized mice were challenged at the same time. Sera were taken 2 days before and 21 days after challenge and titrated for their reactivity with [^{35}S]methionine-labeled glycoprotein material. Symbols: ●, serum samples taken before challenge; ○, serum samples taken after challenge.*

adding rabbit antimouse immunoglobulin M and immunoglobulin G to the reaction mixtures before the addition of Staph A. The results were identical to those shown in Fig. 4.

DISCUSSION

Infection of hairless mice on the snout and forehead with the Tyler strain of HSV-1 caused facial lesions and latent infections of the trigeminal ganglia in more than 80% of the animals, but there was a low (usually less than 10%) mortality rate (21). This model system was used to study correlations between specific immune responses and protection against primary facial and ganglionic infections.

Immunization with live virus in a footpad or with inactivated virus in the intraperitoneal cavity induced long-lasting protection and high levels of neutralizing antibodies (Table 1). Similar results have been reported by others (4, 19, 26, 34, 38). A viral glycoprotein fraction that was prepared by lectin affinity chromatography also conferred long-lasting immunity, but did not induce neutralizing antibodies. The glycoprotein material consisted largely of glycopolypeptides with molecular weights of 120,000 to 130,000 (Fig. 1), and it induced antibodies in mice after immunization that were largely directed against these glycoproteins (Fig. 3). These antibodies are a mixture of at least two polypeptides (41), they are expressed at the cell surface (11; Zweerink and Stanton, submitted for publication), and it has been shown in vitro that they play an important role in the recognition and lysis of infected cells (11, 32). Thus, it is conceivable that glycoprotein-specific antibodies in immunized mice prevented infectious virus from reaching the trigeminal ganglia. This is in agreement with the observation of Waltz et al. (45), who showed that virus-specific antibodies reduced the number of infected ganglionic cells after challenge.

The glycoprotein isolated by other investigators (5, 44) induced neutralizing antibodies, whereas the material described in this study did not. This could be explained by differences in the method of preparation (lectin affinity chromatography or Triton-solubilized infected cells versus material that was obtained after SDS-polyacrylamide gel electrophoresis of denatured virus), in the method of immunization (a single injection of alum-adsorbed material versus multiple injections of material emulsified in Freund adjuvant), and in the animals used (mice versus goats or rabbits).

We considered the possibility that immunization with the glycoprotein material primed the animals for a more rapid synthesis of protective neutralizing antibodies upon challenge. Immunized mice developed neutralizing antibodies 1 day sooner than control mice (Table 2); however, the biological significance of this finding is difficult to evaluate.

Immunization with the glycoprotein material did not seem to prevent virus replication in hairless mice since neutralizing antibodies developed after challenge to approximately the same levels as in control mice (Table 2). However, we cannot rule out the possibility that the small inoculum (2×10^5 plaque-forming units) contained enough antigenic material for antibody induction without viral replication. The spectrum of virus-specific antibodies was much more restricted in immunized mice (Fig. 3, lanes C and D). A suppressive effect was observed in BALB/c (nu/+) mice; neutralizing antibody titers were more than 10-fold higher in control mice compared with immunized mice. It should be noted that neutralizing antibody titers were the same in immunized hairless mice regardless of whether they developed skin lesions.

The role of T-cells in the development of glycoprotein-mediated immunity was investigated by using congenital athymic nu/nu BALB/c mice and their nu/+ littermates. Immunization of nu/nu mice with glycoprotein material did not induce neutralizing or glycoprotein-specific antibodies, but it did protect mice against primary infections (neutralizing antibodies did not develop after challenge in nu/nu mice). Control nu/+ animals developed glycoprotein-specific antibodies before challenge and neutralizing antibodies after challenge, and they were protected against primary infection (Table 3).

These results show that neither glycoprotein-specific nor neutralizing antibodies are essential for protection of nu/nu mice and that the synthesis of these antibodies is probably T-cell dependent. The nonspecific role of macrophages in protection against primary herpetic infections has been established by a number of authors (16, 37), and macrophages in nude mice have increased antiviral activity (29). Furthermore, nude mice have high natural killer cell activity (14). Immunization with glycoprotein material may stimulate macrophages or natural killer cells directly, or it may result in the release of B-cell-specific lymphokines that stimulate these cells (2, 28, 30). Another possibility is the release of interferon after exposure of B-lymphocytes to glycoproteins, with interferon inhibiting viral replication directly or via other mechanisms, such as stimulation of macrophages or natural killer cells (10, 13, 47). Replicating but not ultraviolet-inactivated HSV acts in vitro as a B-cell-

specific mitogen (18, 27). Whether viral glycoproteins have the same mitogenic effect needs to be determined. Presumably, glycoprotein is released from its complex with alum at sufficient concentrations over a 1-month period to exert its nonspecific protective effect.

The results with congenital athymic mice do not exclude the possibility that in hairless mice (and presumably other immunocompetent mice) the glycoprotein-induced protection that was observed for at least 6 months is mediated through glycoprotein-specific antibodies. This would be in keeping with the earlier observation that passive administration of viral antibodies prevents the spread of HSV-2 to the nervous system (33).

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