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Immune Response to Herpes Simplex Virus Infections: Virus-Specific Antibodies in Sera from Patients with Recurrent Facial Infections

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Radioimmunoprecipitation assays were used to identify antibodies against a number of herpes simplex virus type 1-specific antigens in serum samples from individuals with recurrent facial herpes virus infections and from seropositive individuals without recurrent infections. Individuals with recurrent infections contributed three sequential serum samples each: immediately after the appearance of lesions, 3 weeks later, and 3 months later. Antibodies against at least 18 viral polypeptides were present in all positive sera: these included antibodies against the major nucleocapsid polypeptide (approximate molecular weight, 150,000) and against two glycopolypeptides with molecular weights of 115,000 to 130,000. No significant differences were observed between the serum samples in regard to their virus-specific antibody composition. The high-molecular-weight glycopolypeptides were partially purified and used in quantitative titration experiments. All sera tested were equally reactive with this material. It was concluded that under the experimental conditions an individual's susceptibility to recurrent herpetic infections could not be correlated with quantitative or qualitative changes in the levels of virus-specific antibodies.

Infections with herpes simplex virus (HSV) recur despite high levels of neutralizing antibodies (5, 13) and apparent active virus-specific cellular immunity (4, 10). Both humoral and cellular immunities have been shown to be important in preventing infections in experimental animals and humans. In mice, administration of immune serum inhibited the spread of virus from the site of primary infection to the spinal cord (14), and antiviral immunoglobulin G (IgG) prevented the appearance of infectious virus in transplanted latently infected ganglia (18). The importance of the cellular immunity in preventing recurrent infections is suggested by the increased incidence and severity of infections among immunosuppressed patients (1, 2, 12, 15).

We have analyzed sequential serum samples from individuals with primary and recurrent genital herpetic infections for HSV type 2 (HSV-2)-specific antibodies (submitted for publication). For this purpose, cells infected with HSV-2 were labeled with [^{35}S]methionine, and cytoplasmic extracts were prepared. Portions were incubated with serum samples, and antigen-antibody complexes were isolated with Formalin-fixed protein A-bearing *Staphylococcus* immunoadsorbent (Staph A) (8). After dissociation in sodium dodecyl sulfate (SDS) and β-mercaptoethanol, radiolabeled antigens were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by fluorography. Viral polypeptides in the immunoprecipitate were indicative of specific viral antibodies in the particular serum sample. The rationale for this approach was that deficits or fluctuations in the levels of certain virus-specific antibodies may be responsible for the incidences of recurrent infections.

It was found that antibodies against the major glycoprotein complex (molecular weight, approximately 130,000) appeared first after infection, followed by antibodies against the major nucleocapsid polypeptide and a polypeptide with a molecular weight of 62,000 and a number of minor polypeptides. No differences were found between sequential serum samples that were taken from the same individual before, during, and after episodes of recurrent infections. However, sera from patients with a high incidence of recurrent infections reacted with a larger number of viral antigens than sera of patients with a low incidence.

The purpose of this work was twofold. First, we extended the studies described above to individuals with facial herpes lesions; second, we compared antibodies in a group of seropositive individuals without recurrent facial infections with those in a group of individuals who expe-

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rienced recurrent facial infections at least twice each year.

MATERIALS AND METHODS

Buffers and solutions. NET buffer contained 0.05 M tris(hydroxymethyl)aminomethane, 0.005 M ethylenediaminetetraacetate (EDTA), 0.15 M NaCl, and 0.1 mM phenylmethylsulfonylfluoride, pH 7.2; NET-T buffer contained NET buffer plus 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 contained PBS with 0.005 M KCl. SP buffer contained 0.01 M tris(hydroxymethyl)aminomethane (pH 6.8), 2% 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 grown in Eagle minimal essential medium (EMEM) plus 10% fetal calf serum (FCS).

The Tyler strain of HSV-1 was obtained from A. Friedman at the Merck Institute for Therapeutic Research. HSV-1 was plaque-purified three times in PRK cells under carboxymethyl cellulose (16), and the viral yield from one plaque was used to infect 10^6 PRK cells at a multiplicity of infection (MOI) of 0.01 plaque-forming units per cell in EMEM without FCS. Virus was harvested 36 to 48 h later, and this was used to prepare a working stock by infecting 5×10^7 PRK cells with an MOI of 0.01.

Clinical isolates were obtained from the vesicular fluid of recurrent facial lesions. This material was added to PRK cells, and when an HSV-specific cytopathic effect developed, the supernatant fluid was stored at −70°C. Virus was plaque purified twice, and stocks were prepared as described above. Alternatively, stocks were prepared without plaque purification by infecting 10^6 PRK cells at an MOI of 0.01.

Virus was plaqued on PRK cells under carboxymethyl cellulose as described previously (16). After 3 days at 37°C, plaques were visible and could be counted without staining.

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

Labeling of infected cells. PRK cells (in 75-cm^2 Falcon flasks) were infected at an MOI of 0.1 in EMEM without FCS. At 16 h postinfection, the cells were washed with PBS and incubated with 5 ml of EMEM lacking methionine per flask. Thirty minutes later, 100 μCi of [^{35}S]methionine (Amersham Corp.; 500 Ci/mmol) 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% EDTA and 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 PRK cells were harvested similarly and stored as pellets containing 3×10^6 cells.

Immune precipitation. Staph A was bought from the Enzyme Center, Inc. It was reconstituted to yield a 10% (vol/vol) suspension and kept at −70°C in 2-ml portions. Before use, the bacteria were washed as described (8) and suspended in NET-T buffer.

Frozen cell pellets (10^6 [^{35}S]methionine-labeled infected cells or 3×10^6 unlabeled uninfected cells) were 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, 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, 1:25 since this was determined experimentally to be in antibody excess) were preadsorbed for 15 min at 22°C with 20 μl of extract prepared from homologous uninfected, unlabeled cells. To this was added 20 μl of preadsorbed labeled extract, and this mixture was incubated for 60 min at 22°C, 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 at $6,500 \times g$ for 2 min and 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, followed by the removal of Staph A by centrifugation and analysis of the supernatant by SDS-PAGE.

SDS-PAGE. Samples were electrophoresed in 10% discontinuous slab gels for 5 h at 110 V (11). Gels were fixed for 30 min in a mixture of methanol, acetic acid, and water (5:1:5) and 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 (3). Molecular weight markers (Pharmacia Fine Chemicals) were phosphorylase b (94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), and trypsin inhibitor (20,100). They were labeled with [^{14}C]acetic anhydride by suspending the content of a Pharmacia vial in 1 ml of PBS (0.3 M; pH 7.2), followed by the addition of 500 μCi of [^{14}C]acetic anhydride (Amersham Corp.; 60 to 120 mCi/mmol). The mixture was incubated for 30 min at room temperature, and labeled proteins were lyophilized after their purification by chromatography on a P10 column (Pharmacia Fine Chemicals). These markers were coelectrophoresed in all gels (their positions are indicated in Fig. 1, 3, and 4 below).

Lectin-affinity chromatography. Cell extracts were fractionated on columns with the lectin from *Lens culinaris* coupled to Sepharose 4B. [^{35}S]methionine-labeled cells infected with HSV-1 (Tyler) (2×10^6) were suspended in 0.5 ml of NET-T buffer. Nuclei were removed, and the cytoplasmic extract was applied to the affinity column with a packed volume of 0.5 ml. First, the column was washed with NET-T,

and adsorbed material was eluted by adding 10 mg each of glucose and α-methyl-D-mannoside per ml to the buffer.

Radioimmunoprecipitation of viral glycoproteins. Glycoprotein material that absorbed to the lectin from *L. culinaris* and that eluted with α-methyl-D-mannoside and glucose (see above) 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 was incubated at room temperature for 2 h. Fifty microliters of Staph A (10% [vol/vol]) was added, and incubation was continued for another 15 min, followed by centrifugation at 6,500 × *g* for 2 min and one wash of the pellet in NET buffer. Radioactivity in the combined supernatants was determined by adding them to 10 ml of Aquasol (New England Nuclear Corp.). Pelleted material was suspended in 0.2 ml of SP buffer without bromophenol blue and added to 10 ml of Aquasol. Percent precipitation was expressed as (counts per minute [cpm] in pellet)/(cpm in pellet + cpm in supernatant).

RESULTS

Sera. Sera were collected from three groups of individuals (Table 1). Ten individuals at Merck & Co., Inc., in West Point, Pa., with many recurrent facial infections (more than two per year over the past few years) donated three serum samples: the first when early facial lesions appeared, the second 3 weeks later, and the third 3 months later. In three individuals, virus isolation was attempted from facial lesions, and the virus isolated was HSV-1 as judged by restriction endonuclease analysis with *Eco*RI and *Hpa*I (9; data not shown). Five individuals at Stanford University Medical Center with facial lesions donated two serum samples: the first at the time of facial lesions (all were positive for virus lesions), and the second 3 weeks later. Sera were also obtained from seven individuals at Merck & Co., Inc., with high neutralizing antibody titers against HSV-1 (256 to ≥2048) and from one individual with a low neutralizing titer (16); none of these individuals had experienced recurrent facial or genital infections for at least 5 years. Control serum was included. Neutralizing antibody titers were high in all sera except the control and the acute sample of individual 12. Clearly, the latter individual experienced a primary infection.

Virus-specific antibodies in individuals with recurrent herpetic infections. Serum samples from individuals 1 through 15 were reacted with [^{35}S]methionine-labeled extracts of PRK cells infected with HSV-1 (Tyler), and the precipitated antigens were separated by SDS-PAGE and visualized by fluorography (Fig. 1). All sera except the control and the acute serum

TABLE 1. *Patients and sera*

Patient no.[a]	Serum[b]	Neutralizing antibody titer
1	A	1,024
	E	>2,048
	L	512
2	A	256
	E	256
	L	256
3	A	1,024
	E	256
	L	512
4	A	512
	E	512
	L	1,024
5	A	256
	E	1,024
	L	1,024
6	A	1,024
	E	256
	L	512
7	A	128
	E	512
	L	256
8	A	512
	E	512
	L	256
9	A	1024
	E	>2,048
	L	>2,048
10	A	256
	E	256
	L	256
11	A	1024
	E	1024
12	A	<16
	E	64
13	A	>2,048
	E	>2,048
14	A	256
	E	256
15	A	512
	E	512
16		256
17		≥2,048
18		512
19		512
20		2,048
21		≥2,048
22		256
23		16
Control		<16

[a] Patients 1 to 10 (from Merck & Co., Inc.) had often-recurring facial lesions; patients 11 to 15 (from Stanford University Medical Center) had facial lesions; patients 16 to 23 (from Merck & Co., Inc.) were without facial and genital lesions for at least 5 years.

[b] A, Acute serum (within 3 days after appearance of lesions); E, early convalescent serum (3 to 4 weeks after lesions); L, late convalescent serum (3 months after lesions).

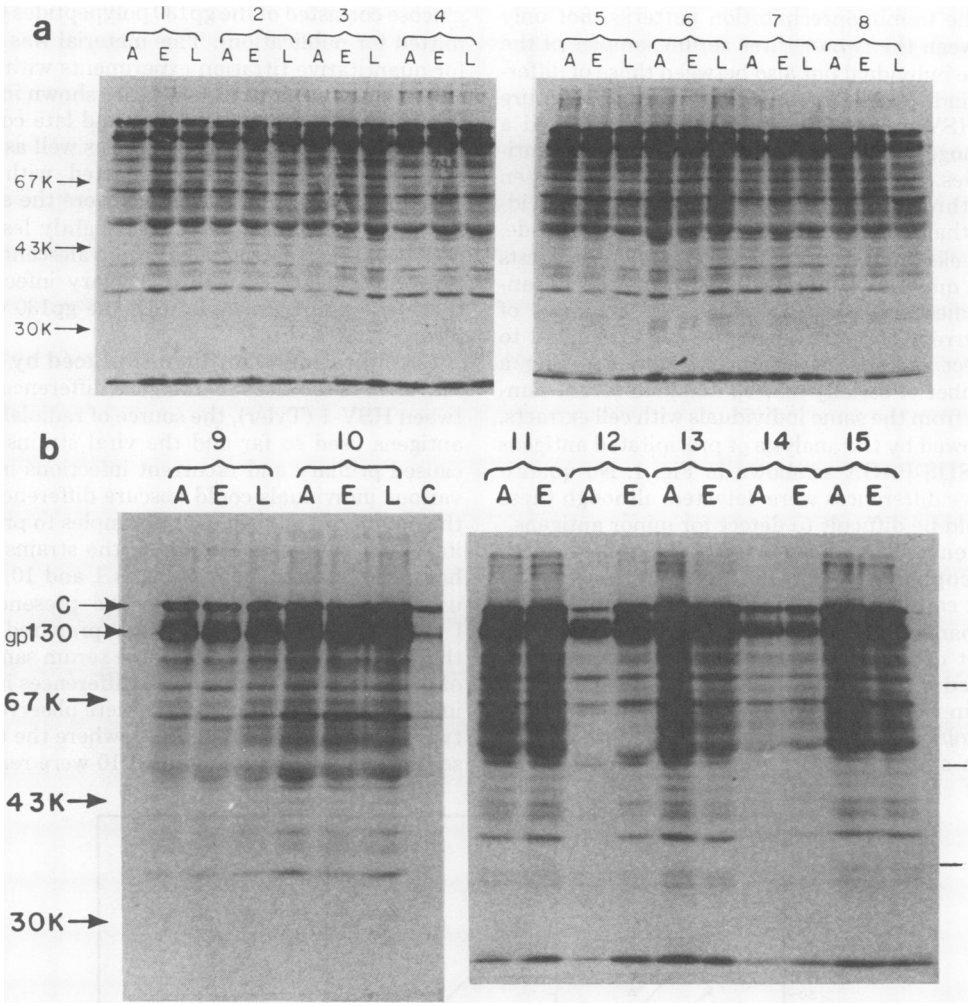

FIG. 1. *Fluorograms of polypeptides precipitated from cytoplasmic extracts of PRK cells infected with HSV-1 (Tyler) by serum samples from 15 individuals with recurrent facial herpetic lesions (see Table 1) and from a seronegative control individual (C). The gel on the right side of (b) was electrophoresed for a shorter time, and the position of the molecular weight markers on this gel are indicated by lines. (a) Patients 1 to 8; (b) patients 9 to 15 and the control. A, Acute serum; E, early convalescent serum; L, late convalescent serum (see the text).*

of individual 12 precipitated more than 18 polypeptides. These were all virus coded since they were not precipitated by control serum (Fig. 1b) and since they were not precipitated from extracts of uninfected cells by immune serum (data not shown). Extracts of infected cells before immunoprecipitation contained many more polypeptides, resulting in gel patterns very similar to those published by others (7). The only precipitated polypeptides that have been characterized are the major nucleocapsid polypeptide with an approximate molecular weight of 150,000 (Fig. 1b) and at least two closely migrating glycopolypeptides with an estimated molecular weight of 115 to 130,000 (gp130) (17; Fig. 1b). Acute serum sample 12 (without neutralizing antibodies) reacted mainly with the gp130 complex, which confirms our earlier observation that antibodies against these glycoproteins are the first ones to appear after a primary infection (submitted for publication). Both acute and early convalescent sera from patient 14 reacted less well with the viral antigens than the other samples, although neutralizing antibody titers were relatively high (256). Otherwise, the most striking feature is the extent of similarity

in the immunoprecipitation patterns, not only between the two or three serum samples of the same individual but also between those of different individuals. Presumably, repeated exposure to HSV during recurrent infections induced a homogeneous population of virus-specific antibodies. Differences were not observed between the three serum samples from the same individual that were taken during a recurrent episode, 3 weeks later, and 3 months later. This suggests that qualitative fluctuations in virus-specific antibodies are not responsible for the onset of recurrent herpetic infections. We attempted to detect quantitative differences by reacting a number of serially diluted matched serum samples from the same individuals with cell extracts, followed by the analysis of precipitated antigens by SDS-PAGE as shown in Fig. 1. No quantitative differences were detected, although these would be difficult to detect for minor antigens.

Reactivity of sera with the major viral glycoprotein complex (gp130). A fraction that consisted largely of the gp130 complex was prepared by fractionating the cytoplasmic extract of [^{35}S]methionine-labeled PRK cells infected with HSV-1 (Tyler) on a column with the lectin from *L. culinaris*. More than 85% of the radiolabeled material that absorbed to the column and eluted with α-methyl-mannoside and glucose consisted of the gp130 polypeptides (submitted for publication). This material was used for quantitative titration experiments with sera 1 to 15. Representative results are shown in Fig. 2. Nearly all sera (acute, early, and late convalescent from the same individual as well as sera from different individuals) reacted with the same efficiency. The exceptions were the acute serum of individual 1 that was slightly less reactive and the acute and early convalescent sera from individual 12 (with a primary infection) that were poorly reactive with the gp130 complex.

Precipitation of antigens induced by clinical HSV-1 isolates. Serological differences between HSV-1 (Tyler), the source of radiolabeled antigens used so far and the viral strains that caused primary and recurrent infections in the various individuals could obscure differences in the ability of matched serum samples to precipitate viral antigens. Therefore, the strains that had been isolated from patients 1 and 10 were used to infect PRK cells in the presence of [^{35}S]methionine. Extracts were prepared and they were reacted with the three serum samples of patients 1 and 10. Again, no differences in the immunoprecipitation patterns were observed. A typical result is shown in Fig. 3, where the three serum samples from individual 10 were reacted

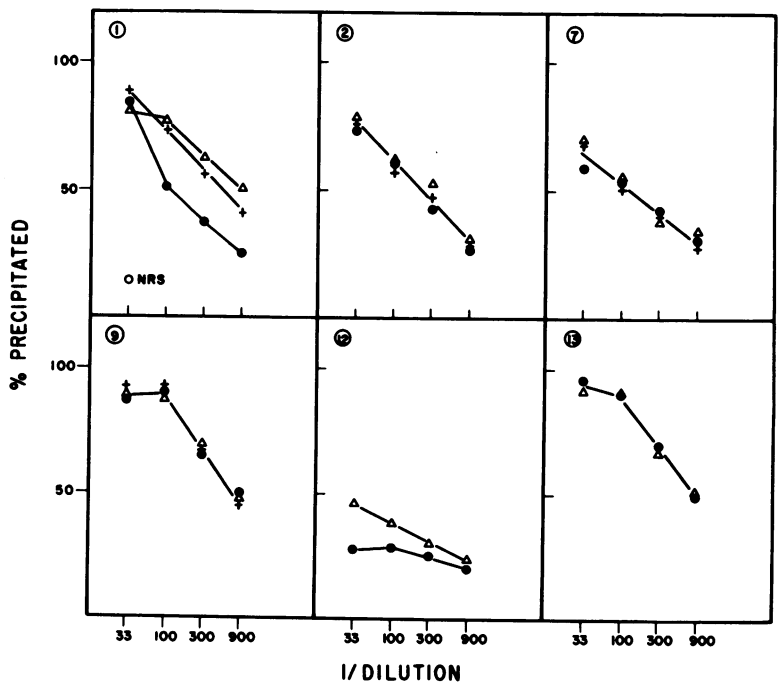

FIG. 2. *Titration of antibodies against the HSV-1 gp130 complex in serum samples from six individuals. The numbers correspond to those in Table 1. Symbols:* (●) *acute serum;* (△) *early convalescent serum;* (+) *late convalescent serum.*

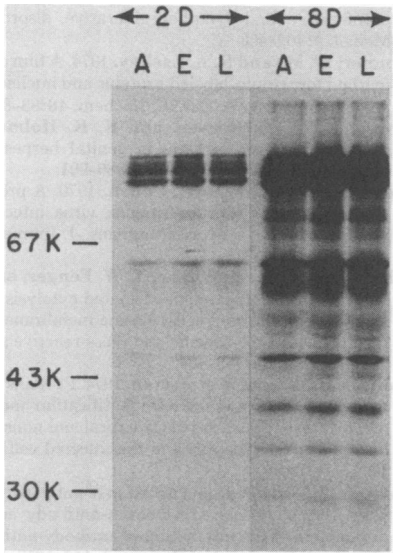

FIG. 3. *Fluorograms of polypeptides precipitated from cytoplasmic extracts of PRK cells infected with the viral isolate from patient 10 with his own acute (A), early convalescent (E), and late convalescent (L) sera. Shown are film exposures of the same gel for 2 and 8 days (D).*

with the antigens of his own isolate. Two different exposure times are shown to highlight minor and major precipitated antigens.

Antibodies in seropositive individuals without recurrent infections. Seven sera with high neutralizing antibody titers (256 to 2,048) and one with a low titer (16) from individuals who never experienced recurrent infections or who had not experienced one over the past 5 years were reacted with extracts from PRK cells infected with HSV-1 (Tyler). For comparison, late convalescent sera from individuals 1, 2, and 3 with recurrent infections were included. Sera were diluted to the same neutralizing antibody titer of 128 (except serum 23 and the control serum, which were not diluted beforehand) and these sera were used in immunoprecipitation assays at a dilution of 1:25. The results of this experiment are shown in Fig. 4. Serum 23 contained mostly antibodies that reacted with the gp130 complex. The other showed little, if any, difference in the composition of virus-specific antibodies. Minor differences (see asterisks in Fig. 4) were sometimes observed but these were not reproducible. Similar results were obtained when the sera were not adjusted to the same neutralizing antibody titer.

DISCUSSION

Antibodies in human sera against individual HSV polypeptides were identified by radioimmunoprecipitation assays. Precipitated radiolabeled viral antigens were identified by SDS-PAGE, and they served to identify specific antibodies. The validity of this assay depends on the absence of aggregates that consist of more than one viral polypeptide species. Previously, we have shown that such aggregates, if present, do not interfere with the assay (submitted for publication). This was determined by reacting cytoplasmic extracts before and after centrifugation at 100,000 × *g* with immune sera and by treating extracts with low concentrations of SDS before the addition of immune sera.

A large number of virus-specific antibodies can be detected in sera from individuals with recurrent facial herpes infections. Two classes of antibodies were identified; those reacting with the major nucleocapsid polypeptide (molecular weight, approximately 150,000) and those reacting with at least two closely migrating glycopolypeptides (gp130; molecular weight, 115,000 to 130,000). The spectrum of viral antibodies was very similar in all sera regardless of whether they were obtained from individuals with frequent facial herpes infections or from seropositive individuals without a history of recurrent infections. Three serum samples were obtained from individuals with recurrent infections: the first at the time of infection, the second 3 weeks later, and the third 3 months later. Again, the antibody composition in these sequential samples did not change.

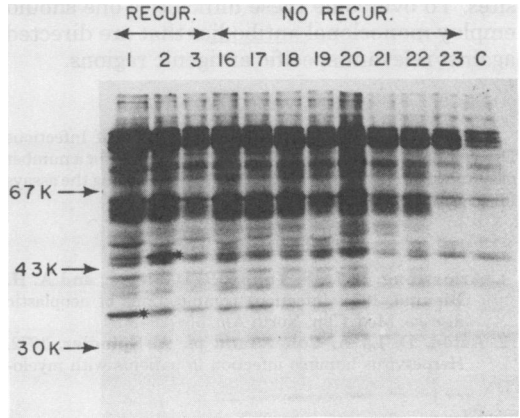

FIG. 4. *Fluorograms of polypeptides precipitated from cytoplasmic extracts of PRK cells infected with HSV-1 (Tyler) by serum samples from patients 1 to 3 (recurrence positive), from patients 16 to 23 (seropositive but recurrence negative), and from a seronegative control (C) (see Table 1). All sera were first diluted to the same neutralizing antibody titer (128) except serum 23 and C, which were not diluted, and 20 μl of each serum was used in the immunoprecipitation assay at a 1:25 dilution.*

The gp130 polypeptides are expressed at the cell surface, and they are believed to play an important role in the recognition and lysis of HSV-infected cells (6). Therefore, levels of antibodies against this glycoprotein complex were tested employing quantitative titration experiments. No differences were found in the levels of antibodies directed against the glycoprotein material. Interestingly, levels of gp130-specific antibodies did not always correlate with complement-dependent neutralizing antibody titers. For example, serum samples from individual 7 differed fourfold in neutralizing antibody titers without changes in gp130-specific antibody titers.

These results suggest that recurrent herpetic infections in susceptible individuals are not caused by temporal fluctuations in the levels of individual classes of virus-specific antibodies and that the absence (or presence) of specific antibodies does not render individuals more susceptible to recurrent infections.

However, one should recognize the limits of the assays that were used for this work. One would expect that antibodies are present that are directed against a large number of antigenic sites on the same molecule. Some of these sites would be more important than others in immune recognition and the suppression of virus replication. The radioimmuneprecipitation assays described in this paper would not detect possible fluctuations in antibodies against these subclasses of immunologically important antigenic sites. To overcome these difficulties one should employ monoclonal antibodies that are directed against relevant specific antigenic regions.

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