

Secretory Immunoglobulin A and Herpes Keratitis

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The sensitization of herpes simplex virus by immunoglobulin G (IgG) antibodies was studied. Although herpes simplex virus sensitized by IgG could not be neutralized by IgA antibodies known to neutralize a similar number of unsensitized viruses, this protection could be overcome by larger amounts of IgA antibodies. This suggests that the effective neutralization of herpes simplex virus in ocular tissue is dependent on the relative concentrations of IgA and IgG antibodies. In vivo sensitized virus was isolated from chronically infected animals, and this may be the mechanism by which herpes simplex virus persists in animals with high titers of circulating antibodies in their serum.

The problem of herpes recurrences has been studied extensively in our laboratory. It was shown by Kaufman (7) that, in the absence of active disease, the virus is chronically released from the ocular tissue of animals with good titers of neutralizing antibodies in their serum (8, 9). A study on the secretory immunoglobulin A (IgA) of the rabbit eye showed several points of interest (5). It was found that topical immunization of the eye with heat-inactivated herpes simplex virus (HSV) led to the production of secretory antibodies of the IgA class. Such antibodies were capable of neutralizing HSV *in vitro* and were also capable of protecting the animal from initial infection independent of serum antibodies. Local immunization of the eye with subsequent production of secretory IgA antibodies did not diminish the number of recurrences in rabbits; however, the level of IgA antibodies in rabbits with and without recurrent disease was similar. Virus could be cultured in the presence of an apparent excess of antiherpes IgA, and experiments indicated that herpes recurrences were not due to a simple deficiency of local antibody production. The question remained how a virus could multiply and be cultured in the presence of neutralizing antibodies of both local and systemic origin.

The apparent resistance of the virus to IgA neutralization and the fact that greater amounts of IgG are found in the tears of diseased animals than in uninfected animals led us to suspect that IgG plays a role in permitting the survival of infectious virus. We felt that this role might be the "sensitization" of HSV by IgG antibodies. In "sensitization," IgG antibody molecules attach to the virus without neutralizing it, presumably coating it so that the subsequent addition of anti-

IgG does result in neutralization (1, 2). Our report deals with the relation between the sensitization of HSV by IgG antibodies and the apparent coating of virus with these antibodies, as evidenced by the resistance of this virus to the neutralizing activity of IgA antibodies.

MATERIALS AND METHODS

HSV. Two strains of HSV were used: the CGA3 strain (McNair Scott; 12) was propagated in HEp-2 cells, and the virulent McKrae strain was maintained in our laboratories in rabbit kidney cells. Plaque assays for HSV in chick embryo fibroblast monolayers were used to quantitate infectious virus as previously described (4).

Antisera. Antiserum to HSV was prepared in rabbits. Animals were infected in the cornea and the disease was allowed to spread systemically. Survivors of this group were further immunized weekly with intramuscular injections of 10^7 plaque-forming units (PFU) of McKrae strain for a 3-week period and were bled at the end of the immunization schedule. A pure antiherpes IgG fraction was obtained by fractionating the serum in a diethylaminoethyl (DEAE) column according to the method of Levy and Sober (10).

Normal goat serum was obtained before immunization, and then anti-rabbit IgG serum was prepared in goats by multiple subcutaneous injections of purified rabbit gamma chain over a 3-week period. An IgG fraction of this serum was also obtained by DEAE column chromatography according to the method of Levy and Sober (10).

The IgA antibodies were prepared from lacrimal secretions of New Zealand white rabbits. The animals were infected in the cornea with the CGA3 strain of HSV and, after the infection was healed, were vaccinated weekly with heat-inactivated virus for a 4-week period. Tears were then collected by carefully inserting a strip of filter paper in the lower lid of the animal's eye for 10 min. The filter papers were eluted overnight

in the cold with an excess of 0.005 M phosphate buffer (pH 7.5), and IgA antibodies were isolated by minor modifications of the method of Cebra and Robins (3). The eluant was applied to a DEAE cellulose column, and the IgA fraction was eluted at 0.3 M NaCl. This fraction was then chromatographed in a Sephadex G-200 column with 0.05 M phosphate buffer (pH 7.5). The IgA fraction was collected in the void volume of the column. The purity of the IgA fraction was tested with goat anti-rabbit α chain and goat anti-rabbit tear serum on Ouchterlony double-diffusion plates, and was found to be free from IgG and other tear proteins. The IgA fraction is presumably an 11S molecule with secretory piece, similar to the one found in rabbit colostrum (3), but the specific type of the antiherpes IgA was not determined in this study. The amount of total IgA and IgG in tear samples was measured by radial immunodiffusion tests on agar plates containing antisera to either rabbit IgA or IgG.

Sensitization and viral assays. To achieve "sensitization," HSV (10^6 PFU) was incubated with an equal volume of antiherpes IgG fraction for 20 min at 37 C. A portion of the original mixture was then combined with equal volumes of a 1:10 dilution of either normal goat IgG or goat anti-rabbit IgG, kept at 4 C for 1 min and then assayed for virus in chick embryo monolayers with the use of three bottles per dilution.

An excess of IgG antibody molecules was determined by centrifugation of the mixture (HSV-IgG antibody) to obtain the supernatant free from virus. This supernatant was incubated with 10^6 PFU of fresh virus per ml, and the titer was reduced to 10^2 PFU/ml. This verified that IgG antibody molecules were in excess in the original mixture.

In several experiments, it was desirable to remove excess IgG antibodies. In these cases, the mixture of HSV plus antiherpes IgG was filtered through a Filter-uge Tube (International Equipment Co., Needham Height, Mass.) with the use of a 50-nm membrane filter (Millipore Corp., Bedford, Mass.) to retain the virus. The filter tube was centrifuged in a clinical centrifuge, and sterile phosphate-buffered saline was then added to remove excess IgG. This procedure was repeated until the filtrate showed no measurable IgG as tested on Ouchterlony diffusion plates with goat anti-rabbit IgG.

The amount of "sensitized" virus in the surviving fraction was determined by subtracting the PFU found after incubation with goat anti-rabbit IgG from those found after incubation with normal goat IgG.

IgA neutralization test. The neutralizing capacity of IgA antibodies was tested on both sensitized and non-sensitized virus. The virus was incubated with an appropriate dilution of IgA antibodies at 37 C for 10 min. A volume of the mixture was pipetted into each of three bottles and gently rolled over the monolayer. Then 2.0 ml of maintenance medium was added, and the bottles were incubated at 37 C for 36 hr. After incubation, the bottles were stained with a crystal violet-acetic acid mixture for plaque counting.

To determine whether "sensitization" occurs in vivo and whether virus recovered from animals was protected from IgA neutralization, the corneas of immune

rabbits were infected with the McKrae strain of HSV, and the disease was allowed to follow its natural course. After the lesions were healed, the cornea and conjunctiva were scraped. The scrapings were placed in minimal essential medium (MEM), frozen at -90 C, and stored.

Serial dilutions of these scrapings were made, a portion was mixed with equal volumes of either goat anti-rabbit IgG or normal goat IgG, and 0.2 ml of this mixture was instilled into each of three bottles of human embryonic kidney monolayers. These were allowed to absorb for 30 min at 37 C and were then overlaid with MEM supplemented with 10% calf serum and 1% methyl cellulose.

RESULTS

Neutralization of sensitized virus by specific IgA antibodies. A sample of HSV (infected HEP-2 supernatant) was sensitized with IgG in the manner described. At the end of the incubation period, properly diluted volumes of virus suspension were mixed with equal amounts of normal goat IgG, goat anti-rabbit IgG, or IgA antibodies from lacrimal secretions; the mixtures were then plaqued in chick embryo monolayers. Although the IgA alone was capable of neutralizing HSV, it failed to do so in the presence of IgG antibodies (Table 1). Further reduction of the plaque count by the addition of goat anti-rabbit IgG indicated that the virus was perhaps complexed with IgG antibody molecules and could not be neutralized by IgA antibody molecules. Whether this was due to simple competition for antigenic sites by the IgG and IgA antibodies molecules was studied in the next experiments.

Determination of the importance of the relative IgA and IgG. To avoid excess IgG molecules in the reaction mixture, it was decided to sensitize the virus in the usual manner, then filter it through a 50-nm membrane filter and wash off the excess IgG molecules as described in Materials and Methods. After filtration, about 95% of the surviving fraction was sensitized. The filtered virus was appropriately diluted and incubated with IgA

TABLE 1. *Antiherpes activity of tear IgA*

Sensitization (20 min at 37 C)	Additive	HSV plaques ^a
HSV + anti-HSV ^b	Normal goat IgG	49
HSV + anti-HSV	Goat anti-rabbit IgG	25
HSV + anti-HSV	IgA	56
HSV	Medium	87
HSV	IgA	43

^a Each value is the average count of plaque-forming units from three bottles.

^b The IgG fraction of rabbit antiherpes serum.

TABLE 2. *Effect of tear IgA antibodies on previously "sensitized" HSV*

Incubation (10 min at 37 C)	Additive	HSV plaques ^a
HSV (10 ⁻¹ dilution)	Normal goat IgG	146
HSV (10 ⁻¹ dilution)	Goat anti-rabbit IgG	2
HSV (10 ⁻² dilution)	Normal goat IgG	17
HSV (10 ⁻² dilution)	Goat anti-rabbit IgG	0
HSV (10 ⁻¹ dilution) + IgA ^b	—	130
HSV (10 ⁻¹ dilution) + IgA (1:2 dilution)	—	122

^a Average count from three bottles.

^b IgA = 1.2 mg/ml.

antibodies for 10 min at 37 C to see the effect of such antibodies on the sensitized virus. Even in the absence of residual excess IgG, the sensitized virus remained resistant to neutralization (Table 2). Since there was a slight decrease in the number of herpes simplex plaques in the mixture containing sensitized virus plus IgA antibodies, it seemed important to find out whether this apparent protection of virus by specific antiherpes IgG molecules was competitive and whether it could be overcome by higher concentrations of IgA antibodies. Virus that was 90% sensitized, filtered, and washed was used. This sensitized virus was incubated with different concentrations of IgA and plaqued in chick embryo monolayers. Results indicated that sensitized HSV could be neutralized in the presence of high concentrations of IgA, and this suggests that protection of HSV from IgA is competitive (Table 3).

IgA and IgG levels in lacrimal gland secretions. IgA and IgG levels in the lacrimal secretions of rabbits were tested both during herpes simplex keratitis and after healing (Table 4). No tear IgG was found in the noninfected animals, but during and after infection significant amounts of IgG were present. In tears of animals subject to recurrent herpes simplex keratitis, both immunoglobulins are interacting with the virion which is chronically released from lacrimal gland and conjunctiva. To study further the persistence of infectious virus, previously infected animals were infected again with the McKrae strain of HSV, and the eyes were cultured after they recovered from apparent disease. Serial dilutions of the specimen were made, and portions were mixed with either normal goat IgG or goat anti-rabbit IgG. These mixtures were titered in human embryonic kidney monolayers. Sensitization of the

virus in vivo by IgG antibodies is indicated by significantly fewer plaques in cultures with goat anti-rabbit IgG than in those with normal goat IgG (Fig. 1).

DISCUSSION

This study was undertaken to investigate several puzzling phenomena: (i) in patients subject to recurrent herpes simplex keratitis (as well as animals previously infected with herpesvirus), virus could be cultured erratically in their tears and saliva over a long period of time (7); (ii) in animal studies, virus appeared to be multiplying in lacrimal gland and conjunctiva (7); (iii) whereas this indicated a continual source of infectious virus, virus could not always be cultured; (iv) tear IgA antibody against herpesvirus had been found in their tears and should have neutralized the virus so that it could not be cultured at all.

The finding of an excess of IgA neutralizing antibody in the tears (11) suggested that herpesvirus might be protected in some way, and prompted us to re-examine the work on "sensitization" to locate any such protective agent. Previous studies of "sensitization" indicated that virus could

TABLE 3. *Effect of different concentrations of tear IgA on "sensitized" virus*

Incubation (10 min at 37 C)	Additive	HSV plaques ^a
HSV + IgA ^b	—	0
HSV + IgA (1:2 dilution)	—	44
HSV + IgA (1:4 dilution)	—	75
HSV + IgA (1:8 dilution)	—	106
HSV + IgA (1:16 dilution)	—	108
HSV	Normal goat IgG	102
HSV	Goat anti-rabbit IgG	30

^a Average count from three bottles.

^b IgA = 2.7 mg/ml.

TABLE 4. *Immunoglobulin level of tears*

Time after infection	IgA	IgG
weeks	mg/100 ml	μg/ml
0 ^a	— ^b	0.0
1	52	35.5
3	47	31.5

^a Noninfected.

^b Present but not in measurable amounts.

be coated by an IgG antibody that did not neutralize it (2). The mechanism for the phenomena of sensitization is still unclear; however, the data presented by Ashe et al. (1) support the hypothesis that sensitized non-neutralized virus in the presence of excess IgG specific antibodies is due to steric hindrance caused by the IgG molecules attached to the virion, and that the addition of smaller Fab fragments could neutralize more virions. This suggests that the small Fab fragments were able to reach antigenic sites on the virion that were not reached by undigested divalent IgG antibody molecules.

The phenomena of sensitization of HSV by IgM antibodies was described by Daniels et al. (6); in this case, the sensitized virus was neutralized by addition of the first and fourth component of complement. It is clear that added complement

is not necessary for IgA neutralization because heat-inactivated IgA neutralizes virus completely. Although added complement might conceivably have enhanced the neutralization by antiherpes IgG in vitro, there was a clear demonstration of sensitized non-neutralized virus in tears in vivo. It seemed possible that this IgG antibody might also protect virus against neutralization by IgA antibodies which are present in tears after acute infections (5).

The results of this study indicate that "sensitization" is a function of the IgG antibody which appears to coat herpesvirus without causing a loss in infectivity. IgG antibody, found in the tears of infected animals, does protect herpesvirus from neutralization by the IgA antibody and, by permitting infectious virus to persist, could lead to recurrences of clinical disease in the ocular or labial region. In vitro, the protection of the virus by IgG from IgA seems to be significant, even when excess IgG molecules have been removed by filtration. Since we have shown that sensitized herpesvirus is found in naturally infected animals and occurs in vivo, a situation exists in which the infectivity of the chronically excreted virus may well depend on the exact relationship between IgA and IgG antibodies, as well as the presence of chronic herpesvirus production. However, these relationships require much greater study, as does the possibility that other herpesviruses may be protected from neutralization in a similar manner.

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LITERATURE CITED

1. Ashe, W. K., M. Magie, and A. L. Watkins. 1969. Kinetics of neutralization of sensitized herpes simplex virus with antibody fragments. *Virology* 37:290-293.
2. Ashe, W. K., and A. L. Watkins. 1967. Kinetics of sensitization of herpes simplex virus and its relationship to the reduction in the neutralization rate constant. *Virology* 33: 613-617.
3. Cebra, J. J., and J. B. Robins. 1966. γ A-immunoglobulin from rabbit colostrum. *J. Immunol.* 97:12-24.
4. Centifanto, Y. M. 1965. A therapeutic antiviral from an extract of λ -infected *Escherichia coli* (phagicin). *Proc. Soc. Exp. Biol. Med.* 120:607-611.
5. Centifanto, Y. M., J. M. Little, and H. E. Kaufman. 1970. The relationship between virus chemotherapy, secretory antibody formation and recurrent herpetic disease. *Ann. N.Y. Acad. Sci.* 173:649-656.
6. Daniels, C. A., T. Borsos, R. Snyderman, and A. L. Notkins. 1969. Neutralization of sensitized virus by the fourth component of complement. *Science* 165: 508-569.
7. Kaufman, H. E., D. C. Brown, and E. D. Ellison. 1967. Recurrent herpes in the rabbit and man. *Science* 156: 1628-1629.

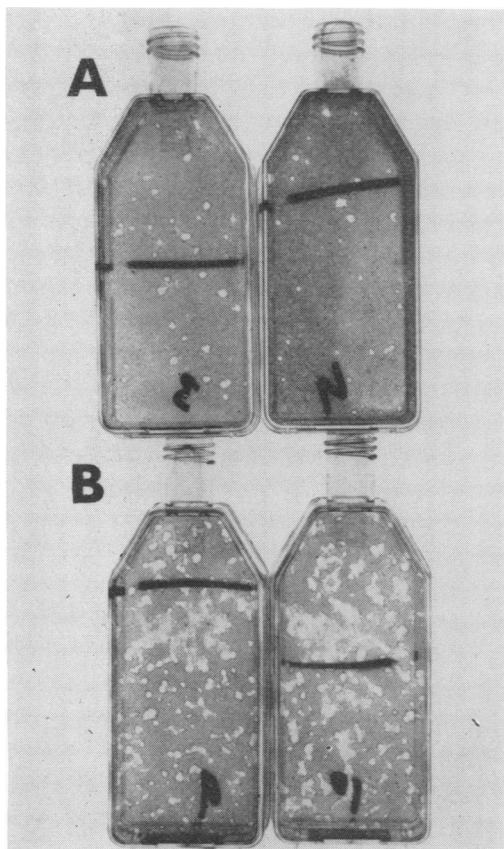


FIG. 1. Herpes simplex virus isolated from scrapings of the conjunctiva and cornea of chronically infected animals which were free from apparent (clinical) disease at the time of culturing. (A) With goat anti-rabbit IgG. (B) With normal goat IgG.

8. Kaufman, H. E., D. C. Brown, and E. D. Ellison. 1968. Herpes virus in the lacrimal gland, conjunctiva and cornea of man—a chronic infection. *Amer. J. Ophthalmol.* 65: 32-35.
9. Laibson, P. R., and S. Kibric. 1969. Recurrence of herpes simplex virus in rabbit eyes. Results of a three-year study. *Invest. Ophthalmol.* 8:346-350.
10. Levy, H. B., and H. A. Sober. 1960. A simple chromatographic method for preparation of gamma globulin. *Proc. Soc. Exp. Biol. Med.* 103:250-252.
11. Little, J. M., Y. M. Centifanto, and H. E. Kaufman. 1969. Studies of immunoglobulin in tears. *Amer. J. Ophthalmol.* 68:898-905.
12. Scott, T. F. McN., and T. Tokumaru. 1964. *Herpesvirus hominis* (virus of Herpes simplex). *Bacteriol. Rev.* 28:458-471.