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Role of Fc Fragments in Antibody-Mediated Recovery from Ocular and Subcutaneous Herpes Simplex Virus Infections

JOHN E. OAKES* AND ROBERT N. LAUSCH

Department of Microbiology/Immunology, College of Medicine, University of South Alabama, Mobile, Alabama 36688

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The contributions of the Fc fragment of virus-specific antibody in the resistance of mice to peripheral herpes simplex virus infection were investigated. Rabbit anti-herpes simplex virus-specific $F(ab')_2$ fragments prepared by pepsin digestion of immune immunoglobulin G (IgG) were found to be inactive in complement-mediated cytolysis while retaining their capacity to neutralize virus infectivity in vitro. When $F(ab')_2$ fragments were passively transferred either before or simultaneously with virus inoculation, they were as efficient as intact IgG was in protecting animals from virus challenge. However, if passive transfer was delayed until 8 h after herpes simplex virus infection, only IgG antibody was protective. The loss of protective activity could not be attributed to a rapid disappearance of $F(ab')_2$ fragments, because comparable levels of $F(ab')_2$ fragments and IgG antibody were maintained in the blood of recipients during the time that antibody mediated its protective effects. The inability of $F(ab')_2$ subunits to activate complement was also not a factor, because complement-deficient A/J mice and complement-sufficient SJL/J mice recovered from herpes simplex virus infection after the passive transfer of IgG. We concluded that the Fc component of the antibody molecule is needed to resolve intracellular infection and that the mechanism by which antibody mediates recovery remains undefined but does not appear to involve virus neutralization or complement activation.

The role of the cellular versus the humoral response in recovery from primary herpes simplex virus (HSV) infection has been the subject of much debate. Initial reports have stressed the importance of cell-mediated immunity in promoting recovery from primary infection (14, 18, 21). However, there is now abundant evidence that antibody can also play an important role (4, 12, 20, 28). Indeed, it is logical to expect that both facets of the immune response can act cooperatively, and recent observations from our laboratory have supported this notion. Specifically, it has been found that the administration of antiviral antibody will facilitate the recovery of mice after corneal HSV type 1 (HSV-1) infection and subcutaneous HSV-2 infection, provided the host also possesses immunocompetent thymus-derived lymphocytes (19). The precise mechanism(s) by which antiviral antibody contributes to host resistance is not known. One possibility is that specific antibody binds to cell-free virus, neutralizing its infectivity and thus reducing virus spread. It is also possible that antibody can destroy virus-infected cells via mechanisms such as antibody-dependent cellular cytotoxicity (7, 13, 24) or complement-mediated cell lysis (1). In the present report, we describe experiments designed to determine whether virus neutralization and complement activation are important mechanisms whereby antibody increases host resistance to HSV. The results indicated that neither mechanism is essential for antibody to promote the recovery from primary herpesvirus infection.

MATERIALS AND METHODS

Viruses and infection of mice. HSV-1 (strain KOS) and HSV-2 (strain 333) originally obtained from Fred Rapp (The Pennsylvania State University College of Medicine, Hershey, Pa.) have been maintained in our laboratory by passage on Vero cells (Flow Laboratories, Inc., Rockville, Md.) at a multiplicity of infection of 0.1 plaque-forming unit (PFU) per cell. The growth medium consisted of minimal essential medium supplemented with antibiotics, 5% newborn calf serum, and $NaHCO_3$. Virus was harvested from infected cells by three cycles of freezing and thawing. The lysate was clarified by centrifugation at $1,000 \times g$ for 10 min and assayed on Vero cells for PFU as previously described (19).

BALB/c mice (Leo Goodwin Institute, Fort Lauderdale, Fla.) were anesthetized with 0.004 mg of pentobarbital per g of body weight. The right eye was then sacrificed by three twists with a corneal trephine. A volume of 0.01 ml of minimal essential medium

containing 3.8×10^7 PFU of HSV-1 was dropped onto the surface of the cornea and massaged into the eye with the eyelid. SJL/J mice (Jackson Laboratories, Bar Harbor, Maine) were infected subcutaneously in the right rear footpad with 10^4 PFU of HSV-2. The pathogenesis of HSV-1 and HSV-2 in these mouse strains after ocular or subcutaneous inoculation in this manner has been described (3, 6, 17). Young adult A/J mice were obtained from Jackson Laboratories. These mice are deficient in the fifth component of complement (15).

Preparation of IgG and F(ab')$_2$ fragments. Rabbit hyperimmune antisera to both HSV-1 and HSV-2 were prepared by multiple intravenous (i.v.) inoculations of virus into 4- to 6-month-old New Zealand white rabbits (5). Serum from each rabbit was harvested by cardiac puncture, heat inactivated, and stored at −70°C.

Gamma globulin from hyperimmune rabbit serum was precipitated with ammonium sulfate (16). Immunoglobulin G (IgG) was isolated from the gamma globulin by affinity chromatography with protein A-Sepharose CL-4B (Pharmacia Fine Chemicals, Uppsala, Sweden) as described by the manufacturer. The F(ab')$_2$ fragment of IgG was produced by pepsin (Sigma Chemical Co., St. Louis, Mo.) digestion of IgG (1 mg of pepsin per 100 mg of IgG) in 0.1 M sodium acetate buffer, pH 4.0, at 37°C for 18 h by a modification of the technique of Nisonoff et al. (16). After incubation, the pH was adjusted to 7.2 with 1 M NaOH. Undigested IgG was removed from the digestion mixtures by protein A-Sepharose CL-4B chromatography. The F(ab')$_2$ fragment was then precipitated with 2 M ammonium sulfate. The precipitate was solubilized in borate-buffered saline (0.2 M boric acid, 0.05 M sodium tetraborate, 0.15 M NaCl), externally dialyzed against borate-buffered saline, and frozen until use at −70°C. Protein concentrations of IgG and F(ab')$_2$ preparations were determined by the method of Lowry et al. (11).

The neutralizing activity of isolated anti-HSV IgG and F(ab')$_2$ was determined by the plaque reduction method described by Rawls et al. (22). In brief, either IgG or F(ab')$_2$ and virus were diluted in minimal essential medium. Volumes containing 1,000 PFU of HSV were added to equal volumes of twofold dilutions of IgG or F(ab')$_2$ preparations. The mixtures were incubated for 1 h and assayed for infectious particles as previously described (20). Neutralizing activity was expressed as the reciprocal of the highest dilution of IgG or F(ab')$_2$ preparations causing a 50% reduction in PFU as compared with control plates (23).

Passive IgG or F(ab')$_2$ therapy. Dilutions of IgG or F(ab')$_2$ were made in phosphate-buffered saline immediately before passive transfer so that the neutralizing activity of each IgG preparation was 1:320 and that of each F(ab')$_2$ preparation was 1:640. At selected times postinfection, IgG or F(ab')$_2$ molecules were injected i.v. in 0.3-ml doses. Control animals received normal rabbit serum (NRS).

Statistical analysis of data in virus challenge experiments. Negative-exponential transformation of survival times ($\theta = 0.1$; T = 20) was performed in the virus challenge experiments as described by Liddell (10). The means and variances of survival times for each group were then calculated from the transformed data, and the level of significance between survivors in control groups and survivors in experimental groups was determined by Student's t test. A computer program in FORTRAN IV was obtained from Stephen I. Vas (Toronto Western Hospital, Toronto, Ontario, Canada) to perform the calculations.

Complement-dependent antibody lysis test. Monolayers of PARA-7 cells (9) were infected overnight with HSV-1 (strain 14-012) at approximately 7 PFU per cell. The next day the cells were harvested by trypsinization, and 10^6 infected cells were labeled with 150 μCi of ^{51}Cr (sodium chromate; New England Nuclear Corp., Boston, Mass.) for 60 min at 37°C in a shaking water bath. The cells were then incubated for 3 h in a 37°C CO_2 incubator, washed, and diluted to a final concentration of 2×10^4 cells per ml. To a 0.1-ml cell suspension, 0.1 ml of the desired IgG or F(ab')$_2$ preparation was added, followed by 0.1 ml of diluted guinea pig serum. The guinea pig serum had been preadsorbed with PARA-7 cells. After incubation for 1 h, 0.7 ml of medium was added to each tube. After 10 to 15 min of incubation, the tubes were centrifuged, and 0.5 ml of the supernatant was drawn off and counted. Maximum ^{51}Cr release was determined by the addition of 2.5% sodium dodecyl sulfate. The assays were performed in triplicate, and the data were statistically analyzed using Student's t test. The percentage of lysis was calculated by the following formula: percent cytotoxicity = [(experimental counts per minute − spontaneous counts per minute)/(maximum counts per minute − spontaneous counts per minute)] × 100.

RESULTS

Evaluation of F(ab')$_2$ preparations. F(ab')$_2$ fragments were prepared by pepsin digestion of hyperimmune rabbit anti-HSV IgG. It is known that anti-HSV IgG must contain an Fc fragment to promote complement-mediated lysis of HSV-infected cells (13). Therefore, the effectiveness of the enzyme treatment was evaluated in vitro by determining whether the antibody molecules had lost their capacity to lyse HSV-infected cells in the presence of complement. Table 1 shows that F(ab')$_2$ preparations produced little or no lysis, whereas the target cells were readily lysed by intact IgG molecules. The enzymatically treated IgG antibody, although unable to activate complement, had virus neutralization titers comparable to those of intact IgG (data not shown).

Clearance of IgG and F(ab')$_2$ after i.v. injection into HSV-infected mice. Groups of six HSV-infected mice were injected i.v. with either one 0.3-ml dose of virus-specific IgG or two 0.3-ml doses of virus-specific F(ab')$_2$ given 16 h apart. Two injections of antibody fragments possessing twice the neutralizing activity of intact antibody were given because it is known that antibody fragments have shorter half-lives

TABLE 1. *Cytolytic activity of HSV-specific IgG and F(ab')₂ fractions for HSV-1-infected cells*

Antiserum	Prepn	Guinea pig complement[a]	% Lysis[b]	*P* value
Anti-HSV-1	IgG (1:10)[c]	+	51	<0.001
	IgG (1:100)	+	38	<0.001
	IgG (1:10)	+ (heated)	3	NS[d]
	$F(ab')_2$[e]	+	7	<0.025
Anti-HSV-2	IgG (1:10)	+	39	<0.001
	IgG (1:100)	+	8	NS
	IgG (1:100)	+	3	NS
	IgG (1:10)	+ (heated)	3	NS
	$F(ab')_2$ (1:10)	+	5	NS

[a] Used at a 1:16 dilution.
[b] Target cells were ^{51}Cr-labeled PARA-7 cells infected for 17 h with HSV-1 (strain 14-012). The spontaneous release of ^{51}Cr was 26%.
[c] Serum dilution.
[d] NS, Not significant.
[e] Protein concentrations of undiluted IgG and $F(ab')_2$ were both 6 mg/ml. The neutralizing activities of the preparations were 1:1,000.

in vivo than do IgG molecules (27). Since a xenogeneic source of antibody was used, the clearance of rabbit IgG and $F(ab')_2$ from the blood of mice may not be physiological. However, the levels of neutralizing activity maintained in the blood of HSV-infected mice receiving two $F(ab')_2$ doses were nearly the same as those maintained in the blood of HSV-infected mice receiving IgG (Fig. 1).

Antiviral activity of IgG and $F(ab')_2$ in vivo. It was important to establish that virus-specific $F(ab')_2$ fragments retained their neutralizing capacity in vivo. This was evaluated in two ways. In one experiment, the mice were given $F(ab')_2$, IgG, or NRS i.v. Immediately thereafter, the animals were given a lethal dose of virus by i.v. inoculation. In a second experiment, $F(ab')_2$, IgG, or NRS was individually mixed with a lethal dose of virus, and the virus-antibody mixtures were immediately inoculated subcutaneously into recipients. The number of animals surviving infection was then compared among the various groups (Fig. 2). It was found that the protection conferred in vivo by virus-specific $F(ab')_2$ in these experiments was comparable to the protection conferred by virus-specific IgG. This observation indicated that $F(ab')_2$ given immediately before or simultaneously with HSV was just as effective as IgG was in bestowing protection.

As noted above, previous work has established that IgG antibody given as late as 48 h after virus inoculation can significantly reduce animal mortality (20). The critical question we then asked was whether $F(ab')_2$ has similar activity. Animals were infected with HSV and then challenged 8 h later with $F(ab')_2$, IgG, or NRS. Figure 3 shows that virus-specific IgG readily

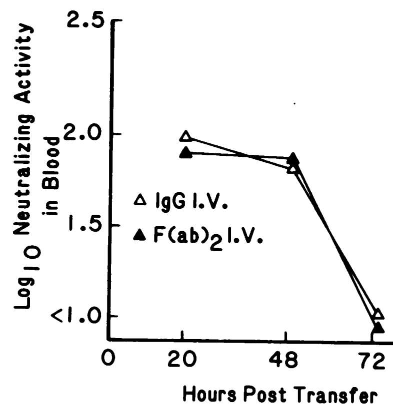

FIG. 1. *HSV-specific neutralizing activity in the blood of mice infected with 10^4 PFU of HSV-2 after i.v. injection of 0.3 ml of anti-HSV-2 IgG (titer, 1:320) 8 h postinfection and $F(ab')_2$ (titer, 1:640) 8 and 24 h postinfection. Each point represents the activity of pooled sera from two mice.*

protected animals from a lethal inoculum of virus ($P < 0.01$). In sharp contrast, virus-specific $F(ab')_2$ had no effect on the course of either ocular or subcutaneous infection. Even when a second injection of $F(ab')_2$ was given to infected animals 16 h after the original treatment, virus-specific $F(ab')_2$ still did not significantly alter the final outcome of virus infection. These results clearly show that the HSV IgG antibody must be intact to be therapeutically effective.

Protective capacity of virus-specific IgG in the absence of complement. Passive immunization of C5-deficient mice was performed to determine whether either the classical or the alternative pathway of complement fixation was involved in the protective effects of IgG in vivo.

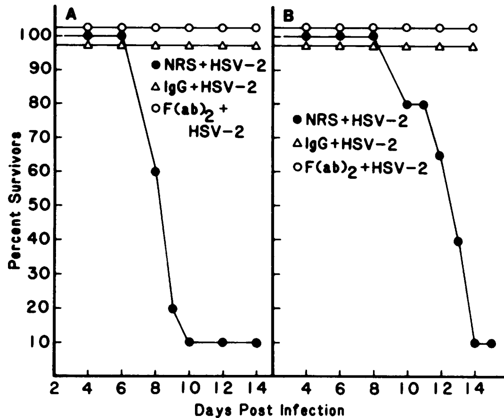

FIG. 2. *Neutralization of HSV in vivo. In (A), animals were given an i.v. injection of 0.3 ml of HSV-2-specific F(ab')₂, IgG, or NRS. Immediately thereafter, they were infected with 10^4 PFU of HSV-2 i.v. In (B), a volume of 0.1 ml of minimal essential medium containing 1.4×10^5 PFU of HSV-2 was mixed with 0.5 ml of either IgG, F(ab')₂, or NRS. Each mixture was then given to groups of mice by subcutaneous inoculation with 0.05 ml of the mixture containing 10^4 PFU of HSV-2 into the right rear foodpad. The number of survivors did not change during 2 additional weeks of observation. There were 10 mice per group.*

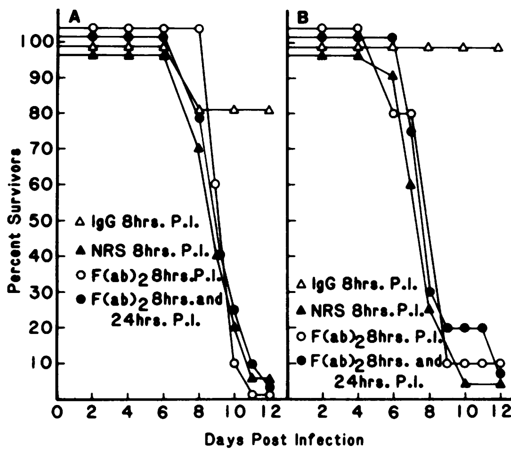

FIG. 3. *Effect of i.v. administration of anti-HSV IgG, F(ab')₂, or NRS on survival of mice after corneal HSV-1 (A) or subcutaneous HSV-2 (B) inoculation. A 0.3-ml volume of either anti-HSV-1 or anti-HSV-2 IgG, F(ab')₂, or NRS was given i.v. at selected times after ocular infection with 3.8×10^7 PFU of HSV-1 or subcutaneous infection with 1×10^4 PFU of HSV-2. The number of survivors did not change during 2 additional weeks of observation. There were 10 mice per group. P.I., Postinfection.*

Groups of complement-deficient A/J mice were infected with a lethal inoculum of either HSV-1 or HSV-2. At 8 h postinfection, a portion of each group was treated with anti-HSV IgG or NRS. It was found that anti-HSV IgG was as effective in protecting A/J mice as it was in protecting complement-sufficient SJL animals (Table 2). Thus, the C5 through C9 components did not appear to be needed for the protective effect. These results suggest that the failure of F(ab')₂ to protect recipients from virus infection is not related to an inability of F(ab')₂ molecules to activate the lytic components of complement.

DISCUSSION

This report describes the results of studies designed to probe how antibody facilitates host recovery from HSV infection. One possibility that we considered for explaining the contributions of antibody to host recovery was that antibody neutralized the infectivity of virus in vivo, thereby limiting the spread of infection. If this is true, then F(ab')₂ fragments of IgG which possess the capacity to neutralize HSV should be as effective as intact IgG in promoting recovery (13). Indeed, our initial in vivo tests established that F(ab')₂ fragments given either immediately before HSV infection or simultaneously with the virus inoculum were effective in bestowing protection. This observation suggests that the neutralizing activity of anti-HSV antibody can contribute to the host's defense against HSV infection whenever conditions are favorable for antigen-antibody formation between specific antibody and HSV virions.

Even though it was apparent that both F(ab')₂ and IgG were sufficient to initiate protection when passively transferred to animals either before or simultaneously with virus inoculation, only IgG was effective in promoting recovery when presented to animals 8 h after virus infection. The failure of F(ab')₂ fragments to initiate protection when given several hours postinfection could be accounted for if F(ab')₂ fragments

TABLE 2. *Protective effects of passively acquired anti-HSV antibody in complement-deficient A/J mice*[a]

Treatment	No. of survivors/no. of animals	% Survivors
HSV-1 + NRS	1/10	10
HSV-1 + anti-HSV-1 IgG[b]	8/10	80
HSV-2 + NRS	2/10	20
HSV-2 + anti-HSV-2 IgG[b]	10/10	100

[a] Four-week-old A/J mice were infected on an abrased cornea with 3.8×10^7 PFU of HSV-1 or subcutaneously with 1×10^4 PFU of HSV-2. At 8 h postinfection, animals received 0.3 ml of either NRS or anti-HSV IgG i.v. Survival was recorded 2 weeks after infection.

[b] Differs from HSV + NRS controls ($P < 0.01$).

disappeared from the circulation of HSV-infected recipients before they had an opportunity to interact with virus antigens in vivo. However, several considerations argue against this interpretation. First, $F(ab')_2$ fragments and IgG antibody were inoculated i.v. into HSV-infected animals. Thus, both $F(ab')_2$ fragments and IgG antibody were in a position to immediately leave the circulation and react with either cell-free virus remaining at the sites of infection or with new virus antigens which would have begun to appear on infected cells by 8 h postinfection (25, 26). Second, comparable quantities of $F(ab')_2$ and IgG were maintained in the blood of HSV-infected hosts. Although the data do not indicate the fraction of $F(ab')_2$ and IgG disappearing from the blood as a result of catabolism (27) or what fraction disappeared as a result of binding with virus-specific antigens, it is clear that indistinguishable levels of neutralizing activity were present in the blood of HSV-infected recipients for 48 h posttransfer regardless of whether recipients received $F(ab')_2$ or IgG. Therefore, we concluded that the Fc portion of the antibody molecule is essential in promoting antiviral immunity when specific antibody is given 8 h postinfection. Furthermore, we also concluded that, at this time postinfection, virus neutralization is not the sole mechanism by which antibody promotes host recovery from HSV infection.

It is known that for antibody to promote complement-mediated cell lysis of HSV-infected cells, the Fc component is required (13) (Table 1). Thus, the inability of $F(ab')_2$ fragments to bind complement could account for the lack of protective activity seen with this molecule. To determine whether complement fixation was important in antibody-mediated recovery, experiments were carried out in complement-deficient mice. A/J mice, which have no detectable C5 in their serum (15), were used. It was found that IgG antibodies were as protective in these animals as they were in animals with an intact complement system. Thus, the later steps in the complement cascade involving C5 through C9 do not appear to be needed for antibody-mediated recovery. The possibility that earlier complement-dependent steps through C3 activation may be involved requires further study.

In our initial experiments, we demonstrated that the Fc fragment of virus-specific antibody was not required in host protection against hematogenous virus spread since both $F(ab')_2$ fragments and IgG antibody could combine with virus particles to neutralize virus in vivo. However, HSV does not appear to disseminate from peripheral sites of infection via the blood in mice, but spreads by centrifugal movement along axons or by movement through intercellular bridges (2, 3, 17). Antibody-dependent cellular cytotoxicity and complement-dependent cell lysis are two mechanisms which could explain the requirement for the Fc component of the antibody molecule in host recovery from HSV infection. Both of these immunological mechanisms can destroy cells expressing virus-specific antigens on their surfaces, and therefore they could be effective against intracellular HSV infection in vivo. A role for the lytic components of complement in promoting recovery from HSV infection in vivo could not be demonstrated in our experimental model. However, antibody-dependent cellular cytotoxicity remains an attractive explanation, not only because it leads to the destruction of virus-infected cells, but also because the Fc component of antibody is required for this mechanism to be operative (8, 13). Whether or not this or some other mechanism is in fact responsible for the capacity of antibody to mediate recovery from peripheral HSV infection will require additional experimentation.

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