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# IMMUNOGENICITY OF SUBVIRAL HERPES SIMPLEX VIRUS PREPARATIONS: PROTECTION OF MICE AGAINST INTRAPERITONEAL INFECTION WITH LIVE VIRUS

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*Summary.* — The capability of crude antigen extracts from herpes simplex virus type 1-infected human diploid cells (AM) to induce protection against intraperitoneal challenge with homotypic but heterologous virus in mice was investigated. The administration of repeated doses of AM without adjuvant failed to confer significant protection. But one relatively small dose of AM with complete Freund's adjuvant resulted in a protective effect. A good correlation was observed between the protective activity of AM preparations and the content of neutralizing antigens as determined by the chromium release-inhibition test. Protection was observed even in animals which were free of neutralizing antibody at the time of challenge. The administration of one dose of cyclophosphamide (120 mg/kg body weight) on day 7 or day 14 after immunization depressed antibody formation but had no effect on the rate of protection. These findings seem to corroborate previous observations on the important role of cell-mediated immunity in protection against herpes simplex.

*Key words: herpes simplex virus type 1; subviral preparations; protection of mice*

## *Introduction*

Previously we reported on the extraction of soluble antigens from human diploid cells infected with herpes simplex virus type 1 (HSV-1) (Kutinová *et al.*, 1977) and on the development of a chromium release-inhibition test (CRIT) for determining the content of HSV-1 neutralization antigens in these extracts (Kutinová and Vonka, 1978). We also described the capability of these antigen extracts to induce a neutralizing-antibody response in different animal species (Kutinová *et al.*, 1979). The antibodies were predominantly complement-requiring and remained so even after administration of repeated antigen doses. Injection of the antigen with Freund's adjuvant resulted in enhanced antibody production. The efficiency of the antibody

response was dependent on the antigen content determined by CRIT. Recently, the ability of the experimental vaccine to restrict establishment of latent injection in rabbits (Rajčáni *et al.*, 1980) and to reduce the severity of skin lesions and neurological complications after intradermal inoculation of mice with HSV were demonstated (Šlichtová *et al.*, 1980).

In the present series of experiments the ability of these preparations to protect mice against intraperitoneal challenge with live HSV was examined.

## *Materials and Methods*

*Cells.* The human diploid lung cells (LEP) and rabbit embryo fibroblast cell line (REF) used were the same as in previous experiments (Řezáčová and Barešová, 1969; Roubal and Vonka, 1973).

*Viruses.* HSV-1 (strain KOS), the same as in previous experiments, was grown in LEP cells at a multiplicity of infection (m.o.i.) of 0.1—0.5 plaque forming unit (PFU) per cell. Eagle's minimal essential medium (MEM) supplemented with 5 % heat-inactivated calf serum, 0.075 % $NaHCO_3$ and antibiotics was used for virus stock preparation. Strain KAD, a finger-blister isolate of HSV-1, was kindly provided by Dr. Z. Janda of this Institute. In our laboratory, it was intraperitoneally (i.p.) inoculated into mice and the virus isolated from the lungs of one animal was subsequently plaque-purified three times in REF cells. A suspension of this virus was prepared in REF cells using EPL medium and a m.o.i. of 0.1 to 0.5 PFU/cell. This virus was selected for challenge because in previous experiments it proved to be highly pathogenic for mice on i.p. inoculation (unpublished data).

*Experimental subviral vaccine.* Soluble antigen extracts from HSV-1-infected LEP cells (AM) were prepared and tested for the absence of live virus as described (Kutinová *et al.*, 1979) The antigen content was determined by the chromium release inhibition test (CRIT) and expressed in CRIT units (Kutinová and Vonka, 1978).

*Mice.* White mice (strain H, breed Srbsko), 4—5 weeks old and weighing 9—11 g, were used throughout.

*Immunization procedure.* Mice were inoculated subcutaneously (s.c.) with one or more doses (given at weekly intervals) of AM (0.1 ml per dose diluted in RSB). The amounts in terms of CRIT units administered are indicated in the individual experiments. In most experiments, mixtures of AM with complete or incomplete Freund's adjuvant (FA) were inoculated s.c. (0.1 ml of mixture/dose); control mice were given the adjuvant mixed with RSB or extracts from uninfected cells. At intervals, the mice were challenged i.p. with different doses of KAD virus. At challenge time, 5 mice of each experimental group were bled. Their sera were pooled and examined for neutralizing antibodies (see below). Animals were observed daily for 15 days after challenge and all deaths were recorded. In most dying mice, clinical symptoms of encephalitis preceded death by one or two days. Mice surviving day 15 remained healthy throughout the observation period. In testing the effect of cyclophospamide (CPA), one dose of CPA (VEB Jenapharm, G.D.R., 120 mg/kg body weight) in saline was given s.c., either prior to or at various intervals after AM administration.

*Neutralization test.* Titres of complement-independent ($C^-$) and complement-requiring ($C^+$) antibodies were determined by the tube neutralization test. For details see Kutinová *et al.* (1979).

## *Results*

### *Protection of mice by AM against HSV-1*

In initial experiments, the ability of AM without adjuvant to confer protection on mice against challenge with homotypic but heterologous (KAD virus) HSV-1 was tested. Mice were immunized s.c. with one, two or three doses of AM (100 CRIT units/dose) and challenged with three different doses of KAD virus 14 days after the last AM dose. Little or no protection was seen

**Table 1. Effect of immunization with HSV-1 soluble antigen extracts (AM) mixed with FA on survival of mice challenged with 1000 $LD_{50}$[1]) of KAD virus**

| Immunized with | Day of challenge 7 | 14 | 21 | Neutralizing antibody on day[2]) 14 C⁻ | 14 C⁺ | 21 C⁻ | 21 C⁺ | KAD virus titre[3]) ($log_{10}$ $LD_{50}$/ml) |
|---|---|---|---|---|---|---|---|---|
| AM[4]) + RSB | 7/7[5]) | 7/7 | 6/7 | <7 | <7 | <7 | 14 | |
| RSB | 7/7 | 5/7 | 5/7 | | | | | 5.3 |
| AM + incomplete FA | 7/7 | 4/7 | 6/7 | <7 | <7 | <7 | 8 | |
| RSB + incomplete FA | 7/7 | 7/7 | 6/7 | | | | | 5.1 |
| AM + complete FA | 7/7 | 1/7 | 1/7 | <7 | <7 | <7 | 34 | |
| RSB + complete FA | 7/7 | 7/7 | 6/6 | | | | | 5.5 |

[1]) 1 $LD_{50}$ corresponded to approximately $1 \times 10^3$ PFU as determined in 6—7 weeks old mice.
[2]) At the time of challenge, 5 mice were bled, their sera were pooled and assayed for C⁻ and C⁺ antibodies. The figures indicate reciprocals of the highest serum dilutions still inhibiting the cytopathic effect in 50% of cultures.
[3]) Groups of mice inoculated with control preparations were infected on day 14 with different virus dilutions ($10^{-1}$ — $10^{-6}$); 7 animals were used per virus dilution.
[4]) 40 CRIT units per mouse.
[5]) No. of mice dead/No. of mice inoculated.

even after administration of three doses of AM. Therefore, mixtures of AM with FA were used in subsequent tests.

AM (40 CRIT units) mixed with RSB, incomplete FA or complete FA was administered to groups of mice. Control mice received RSB or complete or incomplete FA without AM. The mice were challenged with 1000 $LD_{50}$ of KAD virus 7, 14 or 21 days after immunization. To check whether susceptibility of mice to the virus did not decrease as a consequence of administering the adjuvant alone, parallel virus titrations in all three groups of control mice (i.e. those receiving RSB alone, RSB with FA and RSB with complete FA) were performed on day 14, using 7 mice per virus dilution. The results are shown in Table 1. Only the administration of AM with complete FA resulted in significant protection, demonstrable on days 14 and 21 but not on day 7. This protection was clearly not due to the adjuvant activity alone, because virus activity was nearly the same in all three control groups. C⁺ neutralizing antibodies were only detected in samples taken 3 weeks after AM

**Table 2. Effect of immunization with HSV-1 soluble antigen extracts (AM) or extracts from uninfected cells on survival of mice challenged with 1000 $LD_{50}$ of KAD virus**

| Immunization material | Survival of mice[1]) | Neutralizing antibodies on day of challenge[2] C⁻ | C⁺ |
|---|---|---|---|
| RSB with complete FA | 16/18 | <7 | <7 |
| AM[3]) with complete FA | 3/20 | <7 | 40 |
| Control antigen with complete FA | 17/18 | <7 | <7 |

[1]) and [2]) For explanations see Table 1.
[3]) 40 CRIT units.

**Table 3. Effect of immunization with different doses of HSV-1 soluble antigens extracts (AM) mixed with complete FA on the survival of mice challenged with different doses of KAD virus on day 21**

| Dose of AM (CRIT units) | $\log_{10}$ $LD_{50}$/ml | Neutralizing antibody on day of challenge[1]) | |
|---|---|---|---|
| | | $C^-$ | $C^+$ |
| 0.1 | 4.5 | — | — |
| 0.32 | 4.2 | — | — |
| 1.0 | 4.0 | <7 | <7 |
| 3.2 | 4.3 | <7 | <7 |
| 10.0 | 2.8 | <7 | <7 |
| 32.0 | 2.3 | <7 | 14 |
| 100.0 | 1.8 | <7 | 14 |
| RSB only | 5.3 | — | — |

[1]) For explanations see Table 1.

administration, with the highest titres determined in animals given AM with complete FA. $C^-$ antibodies were not detected.

As indicated in Materials and Methods, AM represents crude extracts from virus-infected cells. To prove that the protective effect was due to HSV-specific antigens and not to some cell component(s), groups of mice were immunized with AM from mock-infected LEP cells mixed with complete FA and challenged with 1000 $LD_{50}$ of KAD virus on day 21. No protection was seen in these mice, while nearly all animals immunized with AM (40 CRIT units) in parallel experiments were protected (Table 2).

*Relation between protection and AM dose*

Groups of mice were immunized with different dilutions (i.e. different content of CRIT units) of AM mixed with complete FA and were tested for protection against serial tenfold dilutions of KAD virus three weeks later (six mice per challenge dilution). Neutralizing antibodies in the sera of immunized mice were determined at the time of challenge. The results (Table 3) showed that marked protection was still found after administration of 10 CRIT units of AM. It is noteworthy that at the time of challenge no neutralizing antibodies were detected in mice immunized with this dose of AM; they were only detected in sera of animals immunized with 32 or 100 CRIT units.

*Correlation between the level of protection and the content of CRIT units*

Two independently prepared AMs were tested in CRIT and for protective activity in mice. Fig. 1-I indicates that both preparations had comparable contents of CRIT units. Groups of mice were inoculated with different AM dilutions (mixed with complete FA) containing 2.5—40 CRIT units; 21 days later they were challenged with different doses of KAD virus using 7 mice per virus dilution. The curves illustrating the relationship between the degree of protection conferred by the two preparations tested and the contents of CRIT units inoculated (Fig. 1-II) are comparable. This seems to imply that the rate of protection was a function of the CRIT units contents.

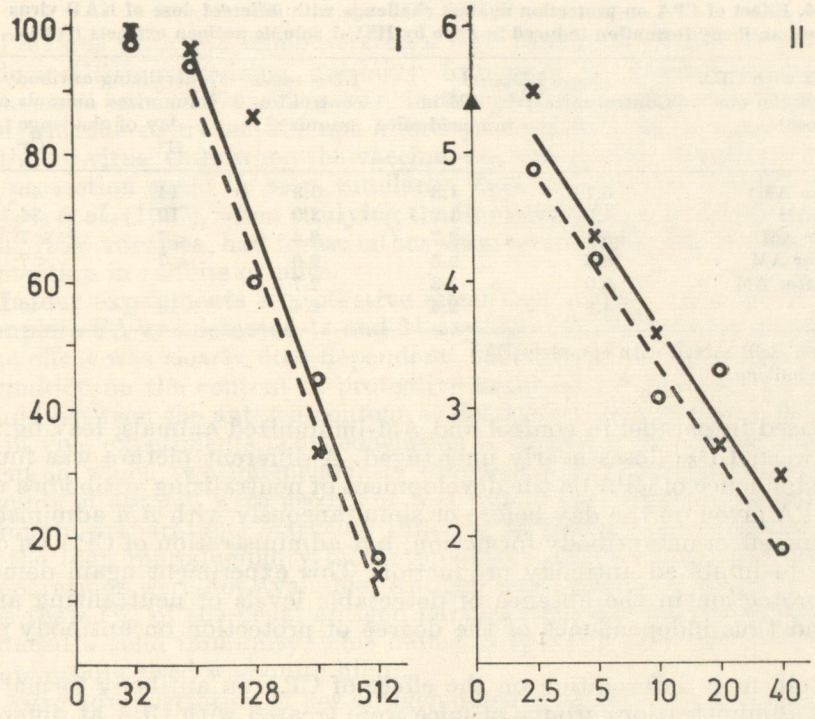


**Fig. 1.**

Correlation between CRIT unit contents and protective activity of two AM preparations

I — Antigenic potency as determined by the CRIT test.
Abscissa: AM dilution reciprocals; ordinate: % inhibition of $^{51}Cr$ release.
II — Protective activity of different antigen doses.
Abscissa: CRIT units inoculated; ordinate: $\log_{10}$ $LD_{50}$/ml.
○------○ $AM_1$, ×———× $AM_2$
▲ Control mice given RSB with complete FA

*Effects of immunosuppression on protection induced by AM*

CPA was administered to mice prior to or after AM inoculation to examine the influence of immunosupression on the development of protection and on antibody formation. Mice were challenged with different virus doses three weeks after immunization with 40 CRIT units. As in previous experiments, sera were taken from five mice in each group at the time of challenge. The results are shown in Table 4. CPA administration did not interfere with the capability of AM to confer protection. CPA administered 7 to 22 days prior to virus inoculation, i.e. one day prior to or 1 to 14 days after AM administration, did not markedly influence the outcome of the virus infection either in control or immunized mice. The infection was only potentiated by the drug in animals which received it simultaneously with the virus, i.e. 21 days after the start of the experiment. However, even under this condition the death

**Table 4. Effect of CPA on protection against challenge with different dose of KAD virus and on antibody formation induced in mice by HSV-1 soluble antigen extracts (AM)**

| Treatment with CPA (120 mg/kg in one dose) | $\log_{10}$ $LD_{50}$/ml | | $LD_{50}$ ratio control immunized | Neutralizing antibody in immunized animals on day of challenge | |
|---|---|---|---|---|---|
| | Control mice[1]) | AM-immunized mice | | C⁻ | C⁺ |
| 1 day before AM | 5.1 | 1.8 | 3.3 | 14 | 40 |
| At time of AM | 4.7 | 1.7 | 3.0 | 10 | 34 |
| 7 days after AM | 5.1 | 2.7 | 2.4 | <7 | <7 |
| 14 days after AM | 4.5 | 2.5 | 2.0 | <7 | <7 |
| 21 days[2]) after AM | 6.0 | 3.3 | 2.7 | | |
| None | 4.8 | 2.4 | 2.4 | 14 | 40 |

[1]) Mice given RSB mixed with complete FA.
[2]) Time of challenge.

toll increased in parallel in control and AM-immunized animals, leaving the ratio between $LD_{50}$ doses nearly unchanged. A different picture was found when the influence of CPA on the development of neutralizing antibodies was tested. CPA given on the day before or simultaneously with AM administration had no effect on antibody formation; but administration of CPA on day 7 or day 14 inhibited antibody production. This experiment again demonstrated protection in the absence of detectable levels of neutralizing antibodies and thus independence of the degree of protection on antibody presence.

To obtain more information on the effect of CPA on antibody formation after AM administration, groups of mice were treated with CPA at different intervals after immunization (with 40 CRIT units). At various time intervals, groups of five mice were bled out and neutralizing antibodies were determined in their sera. The results (Table 5) showed that one dose of CPA, when administered 7 days after inoculation of antigen, completely inhibited antibody formation for at least 6 weeks. When CPA was given 14 days after AM administration, the inhibition was not so marked; antibodies were detected in sera withdrawn on days 28 and 42. Paradoxically, antibody levels were somewhat higher in animals which had received the drug at the time of vaccine administration than in untreated animals.

**Table 5. Effect of CPA on antibody formation induced in mice by HSV-1 soluble antigen extract (AM)**

| Treatment with CPA | Neutralizing antibody[1]) at the indicated day after AM administration | | | | | |
|---|---|---|---|---|---|---|
| | 7 | 14 | 21 | 28 | 35 | 42 |
| None | <7 | <7 | 10 | 24 | 20 | 33 |
| At time of AM[2]) | <7 | <7 | 34 | 57 | 67 | 24 |
| 7 days after AM | | <7 | <7 | <7 | <7 | <7 |
| 14 days after AM | | | <7 | 12 | <7 | 14 |

[1]) For explanation see Table 1; only C⁺ antibody titres are given.
[2]) 40 CRIT units/mouse.

*Discussion*

Previous tests had demonstrated a high capability of AM preparations to induce HSV-neutralizing antibody formation in various animal species (Kutinová *et al.*, 1979). However, in the present experiments three doses of AM without adjuvant did not afford protection to mice against challenge with live virus. Only when the vaccine was mixed with complete FA, a marked protection could be seen. Similarly, Zaia *et al.* (1975), Cappel (1976) and Kitces *et al.* (1977), when studying the immunogenic activity of experimental split HSV vaccines, had to use either alum or complete FA in order to obtain protection in rabbits or mice.

In our experiments a protective effect induced by one dose of AM with complete FA was detected 14 and 21 days but not 7 days after immunization. The effect was clearly dose-dependent. CRIT seems to provide a reliable information on the content of protective antigens, since good correlation was found between the antigen content as determined in this test and the results of in vivo experiments. This conclusion is based on the results of an experiment in which two independently prepared AMs possessing comparable amounts of antigen were used. For this test it would be more appropriate to compare preparations markedly differing in this respect; however, because of the high reproducibility of the procedure used for AM production, such preparations were not available. Antigen doses as low as 10 CRIT units, i.e. less than 1% of the usual antigen harvest from one 1200-ml bottle culture, induced a solid immunity. This indicates that the antigenic potency of the preparations used was quite high.

After administration of low antigen doses, a protective effect was seen even in animals which had been free of detectable levels of neutralizing antibody at the time of challenge. To investigate this point further, the influence of CPA, a substance shown to possess a more severe and longer effect on B than T lymphocytes (Turk and Poulter, 1972), was examined. CPA markedly influenced neutralization antibody formation. This effect was most marked when the drug was applied 7 days after immunization; in these animals no antibody was detected throughout the observation period. Nevertheless, CPA exhibited no marked effect on the protection afforded by AM. These results are consistent with the previous findings demonstrating a significant role of cell-mediated immunity in the control of HSV infection (Mori *et al.*, 1967; Ennis and Wells, 1974; Oakes, 1975; Rager-Zisman and Allison, 1976; Nagafuchi *et al.*, 1979).

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