THE ROLE OF ANTIBODY, DELAYED HYPERSENSITIVITY, AND INTERFERON PRODUCTION IN RECOVERY OF GUINEA PIGS FROM PRIMARY INFECTION WITH VACCINIA VIRUS

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The recovery of animals from primary virus infections may be explained by several possible mechanisms. These include both immune responses, such as antibody and delayed hypersensitivity, and nonimmune mechanisms, such as interferon, fever, and local changes in pH and oxygen tension.

Recent evidence has permitted partial evaluation of the role of the immune responses. Irradiated guinea pigs in which no detectable neutralizing antibody could be demonstrated recovered from vaccinia virus infection as rapidly as did normal animals; the irradiated guinea pigs did, however, develop delayed hypersensitivity to vaccinia during recovery (1). Chick embryos, 10 days or older, may recover from a variety of viral infections although they are unable to produce antibody but are able to produce interferon (2). Human hypogammaglobulinemics usually recover from primary virus infection despite a very limited capacity to produce antibodies (3).

Such findings as these have prompted suggestions that delayed hypersensitivity (4, 5) or interferon (6, 7), with or without other non-specific responses (8) may be more important than antibody in recovery from primary virus infections. Therefore, in order to evaluate the role of delayed hypersensitivity, guinea pigs were treated with methotrexate and x-radiation to block the delayed response (9), and recovery from vaccinia virus infection was observed. Results indicated that animals in which delayed hypersensitivity and antibody production were blocked, but capacity to produce interferon preserved, recovered from vaccinia virus infection as rapidly as normal animals.

Materials and Methods

Animals.—Guinea pigs were 200 to 300 gm Hartley albinos.

Viruses.—Vaccinia virus was propagated in monolayer cultures of guinea pig kidney tissue culture (GPK) maintained in medium 199 (10) or in the chorio-allantoic membrane of the embryonated chick egg and stored at −70°C.

Chikungunya virus (arborvirus group A, African strain) obtained from Mr. C. J. Gibbs, Laboratory of Tropical Virology, National Institutes of Health was propagated by intracerebral passage in the newborn mouse. Encephalomyocarditis (EMC) virus was an r mutant (11) prepared in mouse embryo fibroblast cultures.

Infection and Skin Testing.—Guinea pigs were infected by intradermal inoculation of 0.1 ml of a 10^{-1} dilution of vaccinia virus grown on the chick chorion. This consisted of $10^{3.7}$ 50 per cent guinea pig infectious doses. Animals were skin-tested by intradermal injection of 0.1 ml from the virus pool grown in GPK. The skin test antigen had been heated to 60°C for 20 minutes to inactivate the virus and then stored at 5°C. Reactions with erythema greater than 10 mm in diameter at 24 to 48 hours after inoculation were considered positive. In no instance did a reaction occur before 18 hours after inoculation (1).

Guinea Pig Heart Cultures.—Guinea pig heart tissue cultures (GPH) were prepared by trypsinization of embryo or adult guinea pig hearts. These suspended cells were centrifuged at 500 RPM and resuspended with 50 to 100 volumes of Earle's balanced salt solution at pH 7.8 containing 0.5 per cent lactalbumin hydrolysate and 5 per cent calf serum. The resuspended cells were dispersed in 2 ml amounts into test tubes and propagated as stationary cultures at 39°C. Confluent monolayers usually formed at 3 to 5 days.

Antivaccinia Antibody Assays.—Serum or extracts of lesions were assayed for antibody to vaccinia virus in monkey kidney or HeLa cell tissue cultures by the highly sensitive plaque neutralization test (12). Results were expressed as percentage reduction in number of plaques as compared to controls. In some cases confirmatory tests were carried out using a pox count reduction test on the chorio-allantoic membrane of the embryonated chick egg (13).

Production and Assay of Guinea Pig Interferon.—Guinea pig interferon was produced by infection of GPH tubes with 10^6 to 10^7 plaque-forming units (PFU) of Chikungunya virus (14). Supernatants were harvested 48 to 72 hours after infection. In order to destroy residual virus, it was treated in the presence of 1/100,000 thymol blue with 1 N HCl until pH 2 was reached, transferred to a new flask, and then brought back to neutrality with 1 N NaOH. Interferon was assayed by overnight incubation at 36°C of GPH tubes with 2 ml amounts of material to be assayed. The supernatants were then poured off and cultures challenged with 10 to 30 PFU of EMC virus in 2 ml of Eagle's basal medium containing 10 per cent skim milk (15). After incubation for 1 hour at 36°C a shell overlay (16) containing 0.1 per cent yeast extract, 0.1 per cent bovine serum albumin, 10 per cent skim milk, 1/50,000 neutral red and Earle's balanced salts was applied. A final reading of plaques was made on the first or second day after overlaying.

Guinea Pig Skin Suspensions.—Skin suspensions were prepared by finely mincing guinea pig skin specimens and then grinding the mince in a mortar with sand. The mixtures were then diluted in Eagle's medium and 10 per cent skim milk and assayed as 10 per cent suspensions on chick chorio-allantoic membranes for vaccinia virus content or spun in the ultracentrifuge at 105,000 *g* for one-half hour, filtered through a Millipore HA filter, acid treated, assayed on GPH for interferon or on HeLa cells for antibody.

Methotrexate Dose Schedules.—Aminomethylpteroylglutamic acid was purchased as methotrexate from Lederle Laboratories Division of American Cyanamid Co., Pearl River, New York. Animals were injected intraperitoneally every 48 hours with 5 mg of methotrexate in one cc of Ringer's solution. Treatment was started 2 days before infection with vaccinia virus.

X-Radiation.—Whole body x-radiation of 300 r was administered to guinea pigs by Mr. Henry Meyer of the Radiation Branch of the National Cancer Institute. The dose was given over 2.43 minutes from a 250 kv source using simultaneous dorsal and ventral portals. The average observed mortality in animals receiving both methotrexate and radiation was 10 per cent.

RESULTS

Plan of Experiment.—Four guinea pigs were used in each group. In groups where methotrexate was employed, administration of the drug was begun 48 hours before the animals were infected with vaccinia virus. In groups where animals received x-radiation, it was administered 24 hours before infection. At intervals following infection groups of animals were skin-tested with heat-killed vaccinia virus antigen and the sites read after 6 and 24 hours. The animals were then sacrificed by exsanguination and the site of infection excised.

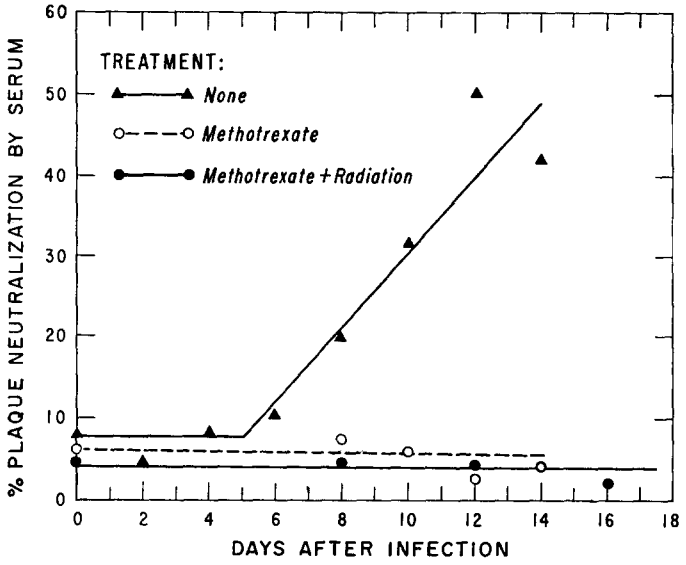

FIG. 1. Serum antivaccinia antibody response in control animals and in animals treated with methotrexate alone or in combination with 300 r total body x-radiation.

The sera were tested for neutralizing antibody and suspensions were made from the skin. Aliquots of the latter were assayed for virus, for interferon, or for neutralizing antibody against vaccinia virus.

Studies on Antivaccinia Antibody in the Serum and at the Site of Infection.—The results of antibody studies on sera taken from experimental animals are shown in Fig. 1. In control groups no significant neutralization of virus by serum was detected before 6 days, and in some experiments as late as 10 days following infection. Circulating antibody steadily increased in titer after first appearing. On the other hand, no significant neutralization of virus was detected in the serums obtained throughout the experiment from animals treated with methotrexate alone or with both methotrexate and x-radiation. In addi-

tion, assay of undiluted samples of 20 per cent skin extracts from lesions excised 1 or 2 days after infection failed to reveal the presence of local antibody in methotrexate- and x-radiation treated-animals. In previous studies no local antibody was present at infection sites of radiated guinea pigs as late as 10 days after infection (1).

Results of Skin Tests for Delayed Hypersensitivity to Vaccinia Virus.—The results of skin tests for delayed hypersensitivity to vaccinia virus are to be seen in Table I. Untreated controls developed delayed reactions on the 5th day following infection with vaccinia.

TABLE I

Effect of Methotrexate Alone or in Combination with 300 r x-radiation on the Development of Delayed Skin Reactions to Vaccinia Virus

Treatment	Number of days after infection with vaccinia virus when skin test performed							
	0	3	5	7	10	12	14	16
None	0/4*	0/4	4/4	4/4	4/4	4/4	4/4	4/4
Methotrexate‡	0/4	0/4	ND§	2/4	1/4	0/4	ND	1/4
Methotrexate and 300 r total body x-radiation‖	0/4	0/4	ND	0/4	ND	0/4	0/3	0/3

* Reported as positives over total tested.

‡ Methotrexate given in 5 mg dose every 48 hours starting 2 days before infection.

§ ND, skin tests not performed on these days.

‖ Total body x-radiation administered 24 hours before infection.

About 75 per cent of the animals treated with methotrexate alone failed to develop delayed skin hypersensitivity to vaccinia virus. There was no apparent difference in the recovery process of those animals which did become delayed sensitive to the infecting agent and those which did not.

In the case of the groups treated with both methotrexate and x-radiation, however, all of the animals failed to develop delayed reactions to vaccinia virus. By studying the course of the vaccinia infection in the immunologically blocked animals, the relative importance of the immune responses could be evaluated.

Course of Vaccinia Infection in Treated and in Untreated Groups.—The course of the vaccinia infection was followed by viral titrations of lesion extracts. It has been previously demonstrated that virus titers remain stable in the infection site over a number of days and that elimination of virus had begun by the 8th day (1). To study the rate of viral elimination, lesion extracts were assayed from the 7th to the 14th day following infection. The course of the infections is shown in Fig. 2, taken from the results of one of several experi-

ments. Untreated controls showed a decline in titer of virus obtained from the skin until no virus was found on the 12th day following the infection, indicating completion of the recovery process. Groups of animals treated with methotrexate alone or with methotrexate and radiation did not significantly differ from controls in that the elimination of detectable virus from the infection site in the skin was complete by two weeks after infection.

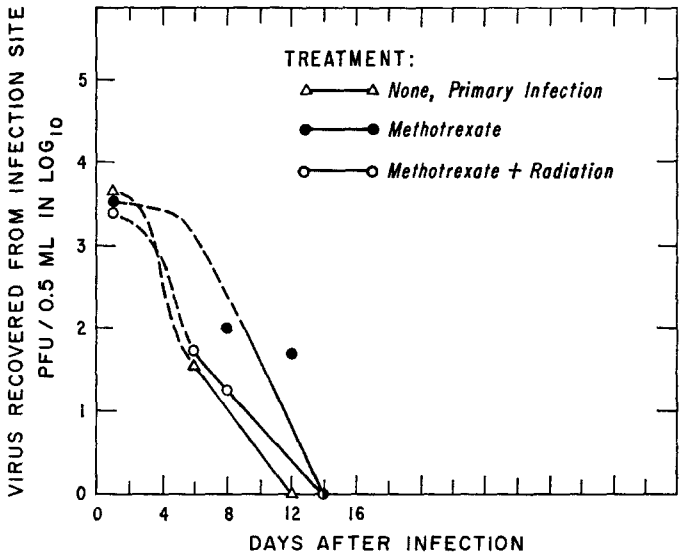

FIG. 2. Titer of vaccinia virus recovered from skin extracts of lesions in control animals and in animals treated with methotrexate alone or in combination with 300 r total body x-radiation. Previous studies (1) demonstrated that virus titers remain stable in the infection sites over a number of days (broken lines).

The results suggested that recovery from primary infection may occur in the absence of delayed hypersensitivity and antibody.

Studies on Infection Sites of Untreated Animals for Production of Interferon.— Since interferon production has been associated with recovery from some virus infections (17–19), experiments were carried out to determine whether interferon was produced during vaccinia virus infection in the skin of guinea pigs. Vaccinia lesion extracts from untreated guinea pigs were acid-treated and ultracentrifuged in order to eliminate residual virus. These extracts were then tested on GPH for their ability to inhibit the growth of EMC virus. As is indicated in Table II, 1/60 dilutions of lesion extracts had greater inhibitory activity on EMC virus plaque formation than did interferon prepared from GPH. On the other hand, as is seen in the same table, skin extracts from uninfected guinea pigs usually showed some enhancement of virus growth when

compared with diluent controls. In other experiments 10 per cent extracts made from inflammatory lesions of guinea pig skin caused by turpentine or by staphylococci had no virus inhibitory properties.

Interferon Production in Infection Sites of Animals Treated With X-radiation and Methotrexate.—As may be seen on Table III, a significant suppression of EMC virus plaque formation was obtained employing skin extracts from the

TABLE II

Suppression of EMC Virus Plaque Formation by Skin Extracts from the Site of Vaccinia Virus Infection in Guinea Pigs

Preparation	Dilution	Number of plaques	Decrease in plaques
			per cent
Diluent control	—	8, 10, 13	—
Guinea pig heart interferon	1/5	1, 1, 4	80
	1/50	8, 11, 15	0
Uninfected guinea pig skin extracts	1/60	17, 17, 19	—
Guinea pig skin extracts:			
48 hrs. after vaccinia infection	1/60	1, 3, 5	82
96 hrs. after vaccinia infection	1/60	6, 10, 15	40

TABLE III

Suppression of EMC Virus Plaque Formation by Skin Extracts from Sites of Vaccinia Virus Infection in Guinea Pigs Treated with Radiation and Methotrexate

Preparation	Dilution	Number of plaques	Decrease in plaques
			per cent
Uninfected guinea pig skin	1/15	32, 42	—
Treated guinea pig skin:			
24 hrs. after vaccinia infection	1/15	0, 5, 8	90
48 hrs. after vaccinia infection	1/15	14, 17	58

site of vaccinia virus infection in guinea pigs treated with 300 r total body x-radiation and methotrexate. The inhibitory factor appeared as early as 1 day after infection and was present on the 2nd day after infection. A skin extract from an uninfected animal treated with x-ray and methotrexate had no virus inhibitory properties.

Some of the properties of the skin extracts prepared from vaccinia infection sites in untreated animals and in animals treated with x-radiation and methotrexate are summarized in Table IV. Similar to the reported properties of

interferon (20) the antiviral activity of skin extracts was stable to acid (pH 2), to 60°C for 1 hour, and had activity in GPH tissue but not in chick embryo cells, the tissue of a heterologous species. Moreover, the antiviral activity was destroyed by boiling, and by trypsin, was not sedimented by 105,000 *g* for one-half hour and was non-specific with respect to virus.

TABLE IV

Properties of Interferon Extracted from Vaccinia Virus Infection Sites of Methotrexate and X-radiation Treated and Untreated Guinea Pigs

Treatment	Antiviral effect following treatment
pH2	+
60°C for 1 hr	+
100°C for ½ hr	0
Sedimentation, 105,000 *g* for ½ hr	+
Trypsin, 0.2 mg/ml for 1 hr	0
Effect on:	
Chick embryo culture (heterologous species)	0
EMC virus (heterologous virus)	+

+, preparation effective.
0, preparation not effective.

DISCUSSION

The present study indicates that guinea pigs in which antibody production and delayed hypersensitivity were blocked recovered as well as normal animals from vaccinia virus infection. Interferon production was present during the course of the infection.

These results are consistent with the hypothesis that, while antibody is important in resistance to secondary viral infections, it is not essential to recovery from primary viral infections (1). The finding that normal recovery occurred in the absence of delayed hypersensitivity suggests that the immune responses are not required for recovery from primary infections with vaccinia virus.

In contrast to the findings in a study of suppression of delayed hypersensitivity to soluble antigens by methotrexate alone (9), complete inhibition of the development of delayed hypersensitivity to the strong antigenic stimulus provided by a replicating virus required treatment with both methotrexate and x-radiation. That treatment with x-radiation and methotrexate in the doses employed does not inhibit vaccinia infection in animals is indicated by the similarity of virus elimination in treated and in untreated groups (Fig. 2) and by the finding that methotrexate did not suppress vaccinia virus infection of rabbits (21). In tissue cultures methotrexate did not inhibit the growth of

vaccinia virus, unless the cells had been made severely thymidine-deficient and even then the suppression was only partial (22).

Additional evidence to support the view that delayed hypersensitivity is not essential to recovery comes from studies of the ability of chick embryos to recover from a variety of virus infections, although the chick embryo is immunologically immature (2). Guinea pigs which had been made delayed hypersensitive to vaccinia virus did not manifest significantly enhanced recovery over controls when both groups were infected with vaccinia virus (23).

There has been increasing evidence that recovery from primary virus infections may be associated with non-immune mechanisms such as interferon (6, 7), elevated temperature (8), changes in pH (8), and decreased oxygen tension (16, 24, 25). In this study the presence of interferon at the site of infection in both untreated and immunologically blocked animals confirms previous work which indicated that interferon was present in vaccinia lesions of rabbits (26) and is consistent with the concept that interferon is an important factor in the recovery. Additional evidence leading to this hypothesis comes from the observation that cell cultures may recover from vaccinia virus infection; recovery was correlated with production of interferon (27).

It seems reasonable that interferon which is active within the cell could be important in recovery from infections with viruses which spread directly from cell to cell without being exposed to antibody (*e.g.* vaccinia, herpes simplex and varicella viruses). There is some evidence that mediation of recovery by non-immune mechanisms may also take place in infections with viruses which spread through antibody-containing extracellular fluids. Older chick embryos which are immunologically incompetent, but which can produce interferon, were found to recover from a variety of virus infections (2). Passive transfer of physiologic amounts of antiviral antibody after the onset of virus infections generally fails to alter the course of the infection (5, 28, 29). Hypogammaglobulinemic humans recover normally from most virus infections despite a markedly reduced capacity to produce antibodies (3). In mice the onset of recovery from influenza pneumonia is better correlated with interferon production than with antibody (17). Inhibition or enhancement of the antiviral action of interferon respectively increases or decreases mortality in mice infected with influenza virus (30). Taken together, this evidence indicates that the non-immune responses alone may be important even in the case of viruses which spread in extracellular fluids.

SUMMARY

In guinea pigs, methotrexate and x-radiation have been employed to block the development of delayed hypersensitivity and of antibody to vaccinia virus during experimental infection.

Animals whose specific responses were blocked by x-radiation and metho-

trexate recovered from vaccinia virus infection as rapidly as non-treated control animals suggesting that these responses were not essential in recovery of the infected animals.

A virus-inhibiting substance with the properties of interferon was present in skin of untreated and methotrexate- and x-radiation–treated animals infected with vaccinia virus.

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