JOURNAL OF VIROLOGY, Feb. 1986, p. 670–673
0022-538X/86/020670-04$02.00/0

Vol. 57, No. 2

Effect of Complement Depletion by Cobra Venom Factor on Fowlpox Virus Infection in Chickens and Chicken Embryos

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Received 24 July 1985/Accepted 11 October 1985

The course of infection with an attenuated strain of fowlpox virus (FPV), which is known to induce antibody-independent activation of complement via the alternative pathway, was investigated in 1- to 3-day-old chickens and 14-day-old chicken embryos by treatment with cobra venom factor (CVF). CVF was found to inhibit complement activity transiently via the alternative pathway but not via the classical pathway. In chickens treated with CVF, virus growth in the skin was enhanced, and pock lesions tended to disseminate, leading to fatal infection in some birds. Histologically, an acute inflammation at an early stage of infection (within 3 days) was inhibited, and virus content in the pock lesion was increased. In chicken embryos with immature immune capacities, CVF treatment caused changes in pock morphology from clear pocks to diffuse ones, an increase in virus content in the pock, and inhibition of cell infiltration. Thus, FPV infection was aggravated in both CVF-treated chickens and chicken embryos. These results are discussed in relation to roles of complement in the elimination of virus at an early stage of FPV infection.

After the first demonstration of antibody-independent activation of the alternative complement pathway by tumor cells (8), cells infected with several viruses have been shown to cause similar complement activation (2, 9, 10). We previously reported (6) that infection of chicken embryo cells with a vaccine strain of fowlpox virus (FPV) causes antibody-independent activation of chicken complement via the alternative pathway, leading to an inhibition of virus growth in vitro. Since deposition of the third component of complement (C3) was demonstrated in FPV-infected skin lesions of the chickens at the progressive stage of lesion development, the significance of complement as an early nonspecific defense mechanism in this virus infection was suggested.

In the present study, chickens and chicken embryos were experimentally depleted of complement by treatment with cobra venom factor (CVF), which inactivates chicken C3 by activation of the alternative pathway, and the course of FPV infection in these birds was investigated to clarify the roles of complement in virus infection in its natural host.

MATERIALS AND METHODS

Chickens. Chicken eggs were purchased from SPAFAS, Inc. (Norwich, Conn.), confirmed to be free from various microbiological infections including FPV, hatched in our laboratory, and raised in an isolator system. The chickens were used at 1 to 3 days of age. Chicken embryos were used at 14 days of incubation.

Virus. A vaccine strain of FPV (Chick-N-Pox; strain no. 946, Gen Corp.) which was previously shown to cause antibody-independent activation of complement was used (6). Stock virus was prepared in cultures of chicken embryo cells, and the infectivity titer of the stock virus was $10^{6.3}$ median tissue culture infectious doses ($TCID_{50}$) per ml.

Treatment with CVF. CVF was purified in this laboratory by the method of Lachmann et al. (4). CVF (500 U) was injected subcutaneously into chickens or into the yolk sac of chicken embryos.

Titration of complement activities. The functional activities of complement via the classical and alternative pathways were titrated by the lysis of sheep erythrocytes sensitized with hemolytic antibody and by the lysis of normal horse erythrocytes, respectively, as described previously (7). Briefly, 50 μl of diluted test serum was mixed with an equal volume of erythrocyte suspension in microtest plates (Nunc, Roskilde, Denmark), and the mixture was incubated at 37°C for 60 min for sensitized sheep erythrocytes and 90 min for horse erythrocytes. The reciprocals of the highest dilutions of serum giving 50% lysis of the erythrocytes were taken as complement titers.

Proliferative response of spleen cells to phytohemagglutinin. Phytohemagglutinin-induced blastogenesis of spleen cells was examined as described previously (11). Spleen cells were suspended in Eagle minimal essential medium supplemented with 2% chicken serum at a concentration of 2×10^6 cells per ml. The cell suspension (0.2 ml) was put into each well of a microtest plate (no. 3506; Costar, Cambridge, Mass.), mixed with 5 to 10 μl of phytohemagglutinin-P (Difco Laboratories, Detroit, Mich.), and cultured at 37°C in a 5% CO_2 incubator for 2 days. Subsequently, 0.1 ml of [^{3}H]thymidine (20 Ci/mmol; Radiochemical Centre, Amersham, England) was added to the cultures, they were incubated overnight, and the incorporation of isotope was counted in a liquid scintillation counter.

Histological examination. The skin lesions of chickens or the chorioallantoic membranes of chicken embryos were fixed in 10% Formalin and embedded in paraffin. The paraffin sections were stained with hematoxylin and eosin stain as usually done.

RESULTS

Effect of CVF on activities of complement and lymphocytes. To determine the conditions of CVF treatment and to confirm its effect on the functions of complement and

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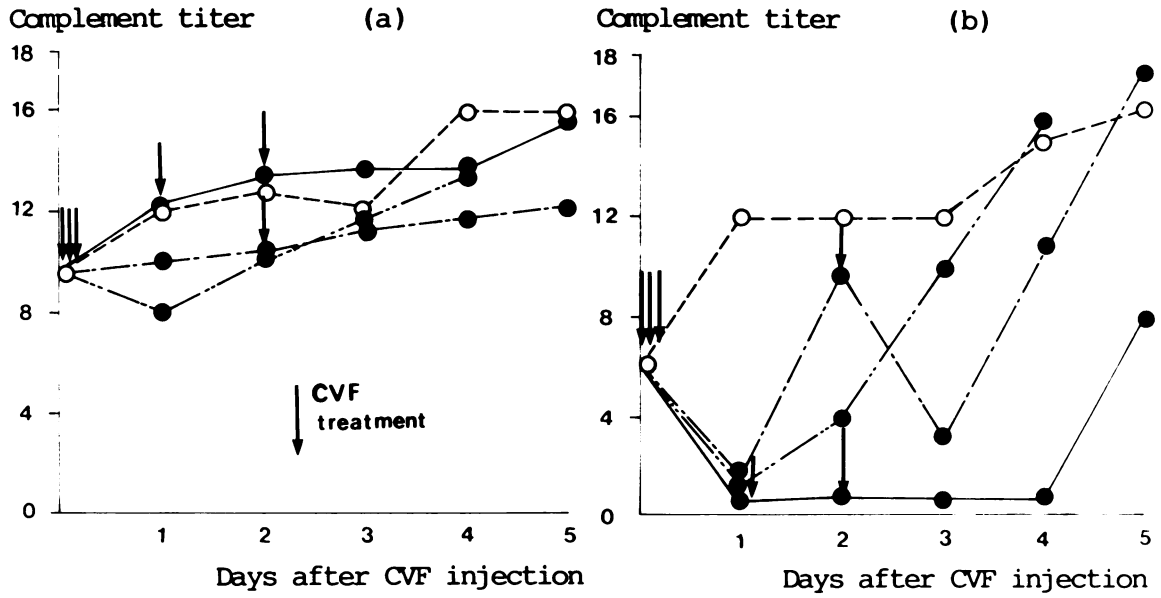

FIG. 1. Functional complement activities measured with either sensitized sheep erythrocytes (a) or normal horse erythrocytes (b) in chickens treated with one dose (●— - - —●), two doses (●— - —●), or three doses (●——●) of CVF or not treated (○).

lymphocytes, we treated chickens with CVF by three different schedules, i.e., one dose, two doses at a 2-day interval, and three doses for 3 consecutive days. The activity of the classical complement pathway was not affected by CVF treatment, whereas the activity of the alternative complement pathway was transiently suppressed (Fig. 1); marked suppression lasting for 5 days was induced by the three-dose treatment. Subsequently, the effect of three doses of CVF on T-cell function was examined by observing the blastogenic activity of spleen cells exposed to the T-cell mitogen, phytohemagglutinin. The same level of stimulation was obtained for the CVF-treated and untreated groups, so T-cell function was probably not affected by CVF (data not shown).

Effect of CVF treatment on clinical course of FPV infection in chickens. Chickens were inoculated with FPV and divided into two groups. One group was treated with CVF on days 1, 2, and 3 after virus inoculation, and the other group was left as an untreated control. The results are summarized in Table 1 and Fig. 2. In the CVF-treated chickens, small diffuse pocks appeared, and all the chickens died on days 7 to 10. In untreated chickens, clearly marginated pocks developed without formation of secondary pocks and disappeared by day 21. Histologically, the pocks in CVF-treated chickens were characterized by hyperplasia of epidermal cells and formation of eosinophilic inclusion bodies. In the untreated chickens, cellular infiltration and degeneration with necrosis and hemorrhages were observed in addition to the hyperplasia and inclusion-body formation. The cellular infiltration consisted of heterophiles, macrophages, and lymphocytes and appeared as early as day 2 in the connective tissues and involved the dermal and epidermal areas on days 3 to 5 (Fig. 2). On day 7, focal necrosis with degeneration of epidermal cells was observed in the centers of the pocks and was surrounded by nodulelike accumulation of lymphocytes and by hemorrhages. The virus content in the pocks was examined at a progressive stage of pock development on day 5. In the pocks in the CVF-treated chickens, virus growth was enhanced 100-fold more than that in the untreated chickens; the titer in the former was $10^{9.5}$ $TCID_{50}$ per g and that in the latter was $10^{7.5}$ $TCID_{50}$ per g.

These results indicate that the depletion of complement causes an enhancement of virus growth and dissemination of pocks so that clinical features are aggravated.

Effect of CVF on FPV infection in chicken embryos. To exclude possible involvement of immune function in analyzing CVF effect on FPV infection, we used as a model 14-day-old chicken embryos, in which both cell-mediated

TABLE 1. FPV infection in CVF-treated chickens

Treatment	Mortality[a]	Skin lesions		Virus titer[c]
		Gross features	Microscopic features[b]	
CVF[d]	10/10 (7–10 days)	Diffuse pocks with dissemination	Hyperplasia; inclusion bodies; degeneration and hemorrhages	9.5
Untreated	0/10	Clear pocks without dissemination	Hyperplasia; inclusion bodies; infiltration of heterophiles; degeneration with focal necrosis	7.5

[a] Number of deaths/number examined. Numbers in parentheses indicate day of death.
[b] Samples were obtained on days 2 to 5.
[c] $TCID_{50}$ per gram of tissue.
[d] Chickens were treated with 500 U of CVF for 3 consecutive days starting from the day of virus inoculation.

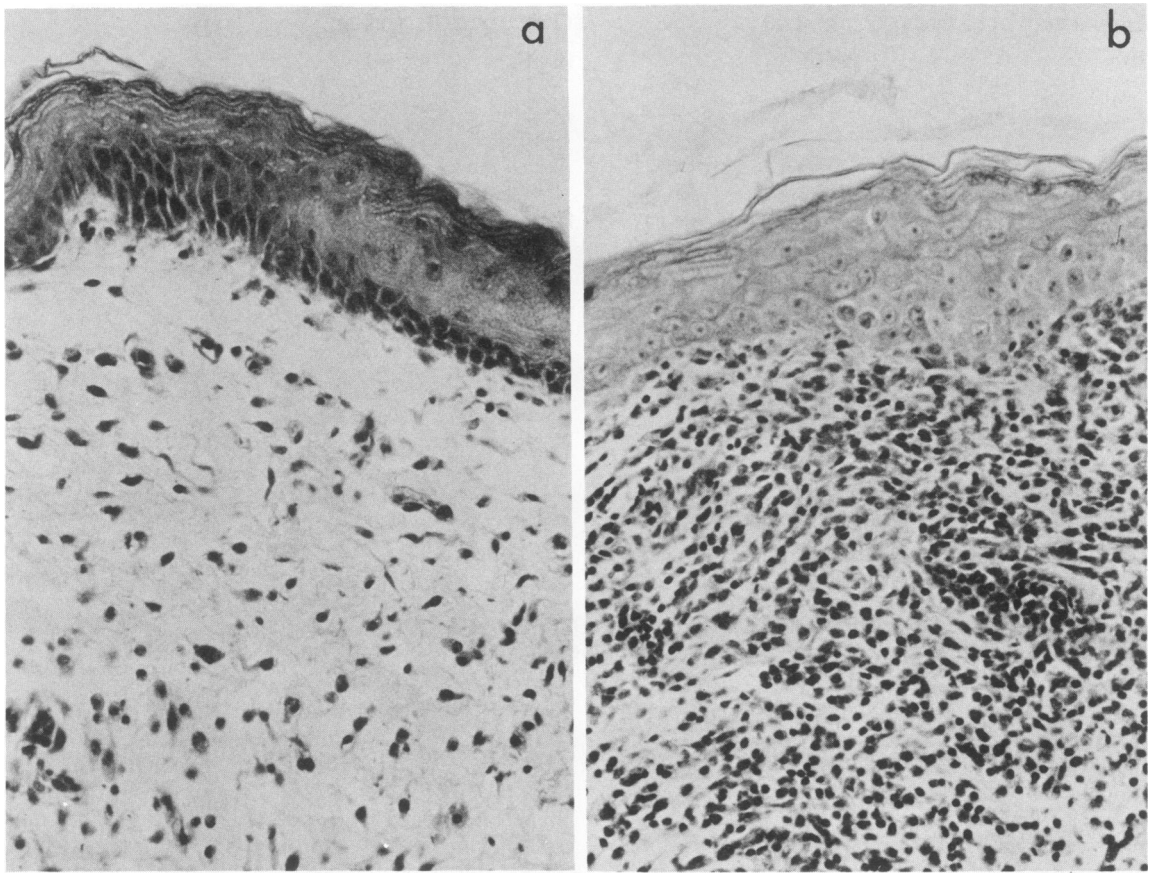

FIG. 2. Histological changes of pock lesions on day 3. (a) Chickens treated with CVF; hyperplasia without inflammatory reactions. (b) Chickens not treated; hyperplasia with severe infiltration of heterophiles. (Hematoxylin and eosin stain; ×200.)

and humoral immunities remain immature while functional complement activities are developed (unpublished data). The embryos were injected in the yolk sac with CVF and inoculated with FPV on the chorioallantoic membranes on the same day. After incubation for 4 days, the chorioallantoic membranes were examined for morphological changes and virus growth. The results are summarized in Table 2. Diffuse pocks with microscopic hyperplasia changes developed in the CVF-treated embryos. In the untreated embryos, clear pocks developed, and their microscopic features were characterized by the infiltration of heterophiles and degeneration in addition to the hyperplasia observed in the CVF-treated embryos. Virus growth was 100-fold higher in the CVF-treated embryos than in the untreated ones. These results are essentially similar to those obtained in the chickens.

TABLE 2. FPV infection in CVF-treated chicken embryos

Treatment	Pock lesions[a]		Virus growth[b]
	Gross features	Microscopic features	
CVF[c]	Diffuse pocks	Hyperplasia	9.5
Untreated	Clear pocks	Hyperplasia; infiltration of heterophiles; degeneration	7.5

[a] Lesions on the chorioallantoic membranes collected on day 4 after FPV infection.
[b] $TCID_{50}$ per gram of chorioallantoic membrane (day 4).
[c] Embryos (14 days old) were injected with 500 U of CVF into the yolk sac and inoculated with FPV on the chorioallantoic membranes.

DISCUSSION

Several viruses are known to activate complement without participation of antibody (2, 9, 10). Thus, the significance of complement as a nonspecific defense mechanism in virus infections has been paid increasing attention. FPV is one of the viruses which cause antibody-independent activation of complement (6). Virus growth and virus-induced lesions are localized in the skin, so the general course of FPV infection can be followed by examination of skin lesions. Moreover, CVF, which is known to deplete mammalian complement by the activation of C3 (1, 5), was found to decrease complement activity of chickens in a preliminary experiment (unpublished data). Similarly, FPV infection can be examined by observing the pocks on the chorioallantoic membranes of chicken embryos in which immune capacity is still immature but the complement system is developed. On the basis of these considerations, the effect of CVF on FPV infection was examined both in chickens and chicken embryos.

CVF was found to decrease complement activity via the alternative pathway, and it was suggested that CVF caused the activation of C3 by this pathway as in the case of the mammalian complement system. Courses of FPV infection in CVF-treated chickens were aggravated in terms of development of pock lesions, virus growth in the lesions, and clinical outcome. The results indicate that depletion of complement was responsible for this aggravation. This con-

clusion was further verified by the finding that similar aggravation was obtained in chicken embryos, in which involvement of cell-mediated and humoral immune responses seem to be unlikely.

It is noteworthy that cellular infiltration into the virus-induced lesions occurred in the CVF-untreated chickens as early as day 2, when the specific immune response has not yet developed. The infiltrating cells consisted of heterophiles, macrophages, and lymphocytes. Cell infiltration was not observed in the CVF-treated chickens. In a previous paper (6), FPV-infected cells were shown to activate the alternative complement pathway. Consequently, it is suggested that the generation of chemotactic factors (3) by the complement activation of virus-infected cells resulted in cellular infiltration. The finding that only heterophiles infiltrated the chorioallantoic membrane lesions of chicken embryos indicates a primary importance of heterophiles in the nonspecific defense mechanisms in the embryonic stage before maturation of the lymphocyte and macrophage system.

One dose of CVF was apparently effective in chicken embryos, whereas three doses were required in chickens. The administered CVF may have persisted longer in the embryos because of the poor capacity of the host to eliminate foreign materials. In this respect, the embryos may be a suitable host for complement-depletion experiments with CVF.

ACKNOWLEDGMENT

This study was supported in part by a grant-in-aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

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