Role of Complement in the Pathogenicity of Fowlpox Virus Infection in Chickens and Chicken Embryos

Hiroaki OHTA, Yasuhiro YOSHIKAWA¹⁾, Kazuya YAMANOUCHI¹⁾, Yoichi ISHIJIMA, Ken-ichi KOMINE, and Hidechika OKADA²⁾

Bio Pharmaceuticals Inc., Gifu Laboratory, Sano-Sotono, Gifu 509-11, ¹⁾Laboratory Animal Research Center, Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo 108, and ²⁾Department of Microbiology, Fukuoka University School of Medicine, Fukuoka 814-01, Japan

(Received 30 July/Accepted 14 October 1987)

ABSTRACT. Chickens and chicken embryos were infected with a vaccine or with wild strain of fowlpox virus (FPV), and the effect of complement activation on the pathogenicity of FPV was examined in terms of histological lesions, mortality, virus growth, and host response. In chicken embryos and in chickens, the complement was activated by the vaccine strain but not by the wild strain. The vaccine strain was avirulent in chickens, whereas it was highly virulent in chicken embryos. Carrageenan treatment that inhibited heterophile infiltration of the pock lesions in chicken embryos reduced the virulence of the vaccine strain. Virus-specific antibody response, as well as protective immunity to challenge from another attenuated strain, developed earlier and at higher levels following inoculation by the vaccine strain than by the wild virus. These findings indicate that complement-mediated reactions are involved in the expression of virus pathogenicity as well as in the induction of immune responses.—**KEY WORDS:** complement, fowlpox, pathogenicity.

Jpn. J. Vet. Sci. 50(1): 145–152, 1988

The significance of complement activation as non-specific defense mechanisms against virus infection has been suggested by several investigators [1, 6, 8, 9]. We previously reported that chicken embryo cells infected *in vitro* with an attenuated strain of fowlpox virus (FPV) activate complement which leads to an inhibition of virus growth without participation of specific antibody production [5]. In a subsequent study [4], depletion of the alternative complement pathway by treatment with a cobra venom factor was shown to aggravate the infection caused by the attenuated FPV resulting in an acute inflammation both in chickens and in chicken embryos. Thus, the complement activation was suggested to play as an early, non-specific defense mechanism [4].

In this study, we compared the clinico-pathogenic courses, including histopathology, C3 deposition, and immune responses, in chickens and chicken embryos which had

been infected with the wild strain or vaccine strain of FPV.

MATERIALS AND METHODS

Chickens. Specific pathogen free-chicken eggs were purchased from SPAFAS (Norwich, Conn.), hatched in our laboratory and raised in an isolator system.

Viruses. A vaccine strain of FPV (Chick-N-Pox, #946 strain) [4, 5], and a wild strain (Nishigawara-FP strain) [3] were used. For the challenge test, another vaccine strain (Poxine strain), obtained from a commercial source (Salsbury Laboratories, Charles City, IO), was used. The infectivity titers of the viruses were determined by the inoculation onto the chorioallantoic membranes (CAM) of 10-day-old chicken embryos and expressed as median egg infective dose (EID₅₀) per ml, base on the appearance of pock lesions of CAM.

Table 1. Characteristics of FPV infections in one-day-old chickens

Characteristics	Virus ^{a)}	
	Vaccine	Wild
Skin lesions;		
Duration (days p.i.)	2-21	2-42
Maximum lesions (days p.i.)	7-10	28-35
Morphology of skin lesions	Thick, clear	Thin, diffuse
Dissemination;	No	Yes
Histological changes (day of appearance);		
Hyperplasia	2	2
Inflammatory cell infiltration	2	28
Virus content in pocks with maximum size (log EID ₅₀)	6.8	9.1

a) Inoculated subcutaneously with 10^{5.3} EID₅₀ of the vaccine or wild strain.

Detection of C3 deposition in the cultured cells or in the lesions. The methods of detecting C3 deposition in cultured cells and lesions were described previously [5]. Briefly, chicken embryo cells were inoculated with vaccine or wild strain at 1 multiplicity of infection, incubated for 2 days, and reacted with fresh normal chicken serum. The C3 deposited in the cells was detected by an indirect immunofluorescent technique. Skin and CAM lesions were frozen and cut by cryostat. The sections were examined by the same method as used for the infected cells.

Histological examination. Samples were collected from skin of CAM lesions, fixed with 10% formalin, and embedded in paraffin. The paraffin sections were stained with hematoxylin and eosin (HE).

Treatment of carrageenan. Five chicken embryos at 9 days of age were injected via the yolk sac route with 5 mg carrageenan (Sigma Chemical Co., St Louis, MO) suspended in saline, and inoculated with the vaccine strain at the same time. The incuba-

tion period was 4 days at 37°C. As control, 5 chicken embryos of the same age were similarly inoculated with the vaccine strain without carrageenan treatment. For histological observation, tissue samples were collected 4 days post inoculation (p. i.).

Antibody titration to FPV. The anti-FPV antibody titer in the chicken sera was determined by an indirect immunofluorescent technique [5]. Briefly, chicken embryo cells infected with the Poxine strain were reacted with sera of various dilutions at 37°C for 1 hr, and further reacted with anti-chicken IgG rabbit serum conjugated with fluorescein isothiocyanate 37°C for 30 min. The antibody titer was expressed as the end-point of serum dilution which showed virus-specific fluorescence.

Resistance to challenge with FPV. One-day-old chickens were inoculated with the vaccine or wild strain, and challenged subcutaneously with the Poxine strain at various days thereafter. Uninoculated chickens at corresponding ages were similarly challenged with the Poxine strain as controls. The sizes of pock lesions at maximum stage were measured for both inoculated and control birds. Degree of resistance was expressed as percent protection;

$$100 - \left(\frac{\text{pock size of control birds} - \text{pock size of test birds}}{\text{pock size of control birds}} \right) \times 100$$

RESULTS

Virulence and histological changes.

The characteristics of FPV infection in one-day-old chickens are summarized in Table 1. In the chickens inoculated with the vaccine strain, pock lesions appeared 2 days post inoculation (p.i.), reached maximum size 7-10 days p.i., and disappeared by 21 days p.i. In the chickens inoculated with the wild strain, pock lesions appeared 2-3 days p.i., plateaued 28-35 days p.i. and were healed by 42 days p.i. In about 20%

Fig. 1. Microscopical features of the skin lesions in chicken. HE staining, $\times 100$. (a) The vaccine strain, 8 days p.i. Severe infiltrations of lymphocytes are observed. (b) The wild strain, 21 days p.i. Only hyperplasia with little infiltration of heterophiles is observed in the both lesions.

chickens inoculated with the wild strain, pocks started to disseminate from 14 days p.i. eventually leading to death. Histologically, the vaccine strain produced the lesions characterized by epidermal cell hyperplasia and heterophile infiltration as early as 2 days p.i. Macrophage and lymphocyte infiltration in the pock lesions was evident after 5 days p.i. Epidermal and dermal degeneration associated with focal hemorrhages in the subcutaneous inflammatory area was observed at this stage. Formation of characteristic follicle-like structures with lymphocyte infiltration was observed 7–8 days p.i. (Fig. 1a). The wild strain caused epidermal cell hyperplasia, but inflammatory reactions were absent except at late stage of infection (Fig. 1b).

In the embryos inoculated onto CAM, virulence in 9- and 14-day-old embryos was estimated in terms of EID_{50} . In 9-day-old embryos, minimum lethal dose (MLD) of

the vaccine strain was $10^{0.1} EID_{50}$, whereas that of the wild strain was $10^{2.7} EID_{50}$. The result indicates that the former is more virulent than the latter. In 14-day-old embryos, MLD of the vaccine strain was $10^{4.1} EID_{50}$ and that of wild strains was $10^{4.7} EID_{50}$, indicating that both strains exhibit similar degrees of virulence. In one-day-old chickens, the MLD of both strains was higher than $10^{5.0} EID_{50}$. These findings show that the virulence of the vaccine and wild strains varies according to the embryo's age. Histologically, characteristic pocks were produced on the CAM by both viruses. The vaccine strain caused ectodermal and endodermal cell hyperplasia, and heterophile infiltration. However, lymphocyte infiltration of the pock lesions was not observed in 9-day-old embryos and occurred rarely in 14-day-old embryos. A large number of diffuse hemorrhages and degeneration of the affected areas was also noticed

Fig. 2. Microscopical features of CAM lesions of the embryos. HE staining, $\times 200$. (a) Vaccine strain: severe infiltrations of heterophiles and hyperplasia are remarkable. (b) Wild strain: hyperplasia without inflammatory reactions is evident.

Fig. 3. Immunofluorescent staining of C3 deposition in vaccine strain-infected skin lesions (3 days p.i.).

(Fig. 2a). The wild strain also caused ectodermal cell hyperplasia, but the inflammatory cell infiltration was observed neither in 9-day-old embryos nor in 14-day-

old embryos (Fig. 2b).

Deposition of C3 in virus-infected chickens and chicken embryos.

Deposition of C3 in skin lesions and in virus-infected CAM was compared for the vaccine and wild strains by an indirect immunofluorescent technique on frozen sections as described previously [5]. Inoculation of one-day-old chickens with the vaccine strain caused C3 deposition at the progressive stage of the lesions, 3 and 7 days p.i. (Fig. 3), but not at the regressive stage, 14 and 21 days p.i. C3 deposition was also observed in the CAM of 9- and 14-day-old embryos inoculated with the vaccine strain. On the other hand, the infection with the wild strain did not cause C3 deposition in any of the skin lesions of one-day-old chickens or CAM of the embryos.

Cultured embryo cells infected with the viruses were also examined for C3 deposition. The embryo cells were inoculated with

Fig. 4. Virus growth in chickens and chicken embryos. Embryos of 9- or 14-day-old or chickens of 1-day-old were inoculated with vaccine or wild strain of $10^{5.0}$ TCID₅₀, and the virus growth in the CAM or skin lesions on various days was determined. Essentially no difference in the virus growth was noted between vaccine and wild strain.

the vaccine or wild strain. After incubation for 2 days, the cells were reacted with fresh normal chicken serum and examined by an indirect immunofluorescent technique similarly. C3 deposition was observed in the cells infected with the vaccine strain but not in those with the wild strain. These results indicate that complement is activated by the vaccine strain but not the wild strain.

Virus growth in the infected lesion.

Growth curves of the vaccine and wild strains were determined in 9- and 14-day-old embryos and in one-day-old chickens after inoculation with $10^{5.0}$ EID₅₀ of the virus (Fig. 4). In 9- and 14-day-old embryos, both strains showed similar growth curves. In the chickens, vaccine virus growth tended to decline after 14 days in contrast to the progressive growth of the wild strain.

Effect of treatment with carrageenan on infection caused by the vaccine strain in embryos.

As an approach to elucidate the mechanisms of high virulence of the vaccine strain in 9-day-old embryos, possible role in the

phenomenon of the acute heterophile infiltration and complement activation, which was observed in embryos infected with the vaccine strain but not in those with the wild strain, was studied. Nine-day-old embryos were injected via the yolk sac route with carrageenan, an inhibitor of granulocyte migration, and inoculated with the vaccine strain. Histologically, carrageenan treatment inhibited the acute inflammation which is normally induced by the vaccine strain. The mean survival time was prolonged by carrageenan treatment, but the virus titers in the pock lesions were similar in both treated embryos ($10^{6.8}$ EID₅₀/g) and untreated ones ($10^{7.3}$ EID₅₀/g). In addition, C3 deposition was evident at the inoculation site of the vaccine strain in the embryos treated with carrageenan. These results indicate that heterophile infiltration induced by complement activation may have caused a detrimental reaction in the embryos in which immune capacity is not well developed.

Development of virus-specific immunities.

Fig. 5. Immune response of one-day-old chickens inoculated with vaccine or wild strain. (a) Antibody titers in sera of the chickens were determined using FPV-infected chicken embryo cells as targets, and expressed as reciprocals of the highest serum dilution showing positive fluorescence by an indirect immunofluorescent technique. (b) Resistance to the challenge with the Poxine strain of FPV. One-day-old chickens were inoculated with the vaccine or wild strain, and challenged subcutaneously with Poxine strain at various days thereafter. Uninoculated chickens at corresponding ages were similarly challenged with the Poxine strain as controls. The sizes of pock lesions at maximum state were measured for both inoculated and control birds. Degree of resistance was expressed as percent protection.

One-day-old chickens were inoculated with the vaccine or the wild strain, and development of specific immune responses was investigated. Antibody response in the chickens inoculated with the vaccine strain occurred earlier and at higher levels than did response to the wild strain (Fig. 5a). Moreover, the resistance to the challenge with the Poxine strain developed earlier in chickens inoculated with the vaccine strain than in those inoculated with the wild strain (Fig. 5b). These results suggest that the vaccine strain induces virus-specific immunities more effectively than the wild strain.

DISCUSSION

In the study, clinicopathological courses in chickens and chicken embryos were compared between the vaccine and wild strain of FPV. The vaccine strain caused

antibody-independent complement activation in chickens, chicken embryos, and cultured chicken embryo cells, whereas the wild strain did not do so in any of these hosts. Histologically, acute inflammation in chickens was induced more effectively with the vaccine strain than with the wild strain.

Previously we showed that suppression of complement in chickens by administration of cobra venom factor aggravate the clinical course. In the decompemented chickens, the FPV-induced acute inflammation was decreased. Thus, acute inflammation induced by complement activation was indicated to act as an early defense mechanism to combat infection of the vaccine strain. The lack of acute inflammation in the chickens inoculated with the wild strain which was shown not to activate C3 demonstrated in the present study is consistent with this interpretation.

On the other hand, higher virulence of the vaccine strain was observed in chicken embryos than the wild strain. Histological observation on the embryos inoculated with the vaccine strain revealed that heterophile infiltration occurred without the subsequent accumulation of lymphocytes. By treating the embryos with carrageenan, heterophile infiltration of pock lesions was markedly suppressed and the surviving time was prolonged by about 2 days. However, virus growth rate, observed mainly in the CAM, was almost the same since a similar virus yield was detected in both the carrageenan-treated and untreated embryos. These results suggest that the early death of embryos by the vaccine strain is due to the side effect of inflammatory infiltration by heterophiles. The heterophiles are known to release cytotoxic substances such as superoxide anions and lysosomal enzymes [10] in response to microorganisms. However, the detoxication system to prevent adverse effect to self cells, such as superoxide dismutase, might not be developed enough in the embryos [2].

It is noteworthy that the challenge inoculation by the vaccine strain led to more effective antibody production and protective immunity than did the wild strain. Inflammatory response, initiated by the vaccine strain-induced complement activation, might have promoted the induction of these specific immune responses to FPV as suggested by the reactions of virus-infected tumor cells [6, 8]. As an alternative, it is possible that the wild strain has an immuno-suppressive effect.

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

REFERENCES

1. Hirsch, R. L. 1981. The complement system: its importance in host response to viral infection. *Microbiol. Rev.* 46: 71-85.
2. McCord, J. M. and Fridovich, I. 1969. Superoxide dismutase: An enzymic function for erythrocyte hemoglobin (Hemocuprein). *J. Biol. Chem.* 244: 6049-6055.
3. Morita, C. 1973. Studies in fowlpox viruses. I. Plaque formation of fowlpox virus on cell culture. *Avian Dis.* 17: 87-92.
4. Ohta, H., Yoshikawa, Y., Kai, C., Yamanouchi, K., Taniguchi, H., Komine, K., Ishijima, Y. and Okada, H. 1986. Effect of complement depletion by cobra venom factor on fowlpox virus infection in chickens and chicken embryos. *J. Virol.* 57: 670-673.
5. Ohta, H., Yoshikawa, Y., Kai, C. and Yamanouchi, K. 1983. Activation of chicken alternative complement pathway by fowlpox virus-infected cells. *Infect. Immun.* 42: 721-727.
6. Okada, H., Okada, N., Tanaka, H., Kohno, K. and Kuroiwa, A. 1984. Suppression of tumor growth in guinea pigs by simultaneous inoculation by tumor cells treated with UV-irradiated Sendai virus at a different site. *Med. Bull. Fukuoka Univ.* 11: 15-17.
7. Okada, N., Shibuta, H. and Okada, H. 1979. Activation of the alternative pathway of guinea-pig complement by Sendai virus-treated cells. *Microbiol. Immunol.* 23: 689-692.
8. Okada, H., Tanaka, H. and Okada, N. 1985. Increased immunogenicity of tumor cells by induction of complement activating capacity of cell membrane. pp 255-270 *In: Basic Mechanisms and Clinical Treatment of Tumor Metastasis* (M. Torisu and T. Yoshida. ed), Academic Press, New York.
9. Sissons, J. G. P., Oldstone, M. B. A. and Schreiber, R. D. 1980. Antibody-independent activation of the alternative complement pathway by measles virus-infected cells. *Proc. Natl. Acad. Sci. USA.* 77: 559-562.
10. Weissmann, G. Smolen, J. E. and Korchak, H. M. 1980. Release of inflammatory mediators from stimulated neutrophils. *N. Engl. J. Med.* 303: 27-34.

要 約

鶏痘ウイルスの病原性発現における補体の役割：大田博昭・吉川泰弘¹⁾・山内一也¹⁾・石島洋一・小峯健一・岡田秀親（ゲン・コーポレーション，¹⁾東京大学医科学研究実験動物研究施設，²⁾福岡大学医学部微生物学教室）—— ニワトリひなあるいはその鶏胚に鶏痘ウイルスワクチン株あるいは野外株を接種して病原性，組織学的変化，ウイルスの増殖性，特異免疫の成立について，補体活性化の影響を検討した．ワクチン株は細胞，鶏胚・ひなのいずれにおいても C3 の沈着を起こすことから，補体の活性化が考えられたが，野外株では補体活性化はみられなかった．ワクチン株接種ひなでは善感発痘がみられ，鶏胚での症原性は野外株より高かった．偽好酸球浸潤を防止する carrageenan で鶏胚を処置してワクチン株を接種したところ，処置しない鶏胚にくらべて低い病原性と示した．ワクチン株接種では，野外株の場合と比較して，他のウイルス株攻撃に対する抵抗性，特異免疫の成立が早く，より高いレベルに達した．これらの成績から，鶏痘ウイルス感染により誘導される補体活性化が病原性の発現および特異的免疫の成立に関与することが示唆された．