Viral and Cellular Growth and Sequential Increase of Protein and DNA during Fowlpox Infection *in Vivo** (32700)

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Fowlpox virus infection in fowls, the specific host, is characterized by pronounced but transitory hyperplasia of cutaneous epithelium with development of characteristic intracytoplasmic viral inclusions (1,2). The development of fowlpox hyperplasia and inclusion bodies in chick chorioallantoic membrane and chick skin (1,3,4) have been repeatedly confirmed as prominent features of infection in these systems (Randall, unpublished observations) and extended with finer resolution by electron microscopy (5,6).

Experiments were undertaken in this laboratory to investigate biochemical events associated with the development of the hyperplastic lesion of fowlpox. Although the use of *in vitro* systems for examining the biochemistry of an animal virus infection has many obvious advantages, neither chick epithelial cell cultures (7) nor short term cultures of chick embryo cells (Randall and Gafford, unpublished data) infected with fowlpox virus exhibit significant hyperplasia or inclusion development. Since *in vitro* induction of hyperplasia appears unlikely, quantitative techniques are being developed to study this poorly understood but important response to viral infection in chick epithelium infected *in vivo*.

The present communication describes viral and cellular growth and compares the sequential content of total protein and DNA in control and infected tissue.

Materials and Methods. Inoculation and collection of tissue. The skin of the scalp and neck of 1-day-old cockerels was inoculated with the Doll strain of fowlpox virus 2 hours after plucking according to Randall *et al.* (8). Control and infected animals were kept in separate heated cages under conditions of continuous lighting and free access to starter ration and water.

As fowlpox infection is characteristically epithelial, it was desirable to restrict analyses to more accessible cutaneous squamous epithelium (referred to as surface epithelium) and to disregard follicular epithelium which, if infected for fewer than 5 days, cannot be effectively separated from connective tissue. Excised sections of skin from groups of animals killed at selected intervals with chloroform were either fixed in 10% neutral buffered formalin for sectioning or washed and partitioned into the desired epithelial and connective tissues by the trypsin procedure of Randall and Gafford (9). In order to quantitatively compare our results utilizing the *in vivo* system in terms common to both control and infected tissue throughout the experiment, preparations of surface epithelium for analyses were obtained from sections of skin measuring approximately 5 cm in length $\times$ 1.5 cm in width (7.5 cm^2 surface area). The results of all determinations were expressed relative to this constant sample. The thickness of epithelium was not included in the determination of sample size because this dimension increased during infection (see Table I).

Histology and enumeration of epithelial cells. Determination of cellularity by the conventional technique of enumeration in counting chambers proved unsuccessful due to technical difficulty in the dispersion of this tissue into suspensions of single cells. A method was therefore devised to assess the kinetics of cellular proliferation by direct microscopic examination. Chick scalps fixed in 10% neutral buffered formalin were embedded in paraffin, sectioned uniformly at 5μ, and stained with hematoxylin and eosin. Care was taken to avoid cutting sections at oblique angles. Sequential slides of control and infected skin were examined with a microscope fitted with a Bausch and Lomb ocular mi-

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TABLE I. Sequential Increase in Thickness and Cellularity of Chick Epithelium Infected *in Vivo* with Fowlpox Virus.

Postinoculation (hours)	Thickness of epithelium (mm × 10^{-2})		Epithelial cells/sample[a] (× 10^5)	
	Control	Infected	Control	Infected
0	1.10 ± 0.07[b]	0.60 ± 0.06	3.60 ± 0.16	3.75 ± 0.10
12	1.00 ± 0.06	0.60 ± 0.05	3.60 ± 0.12	4.05 ± 0.11
24	1.20 ± 0.10	1.30 ± 0.06	3.60 ± 0.14	4.05 ± 0.10
36	1.00 ± 0.06	1.20 ± 0.05	4.05 ± 0.10	3.90 ± 0.12
48	1.10 ± 0.07	2.70 ± 0.09[c]	3.75 ± 0.14	5.55 ± 0.14[c]
60	1.10 ± 0.05	5.40 ± 0.17[c]	4.05 ± 0.11	8.10 ± 0.26[c]
72	1.40 ± 0.09	8.50 ± 0.16[c]	4.20 ± 0.14	9.60 ± 0.23[c]
84	1.50 ± 0.09	8.40 ± 0.28[c]	4.20 ± 0.12	9.30 ± 0.23[c]
96	1.40 ± 0.09	8.10 ± 0.34[c]	4.20 ± 0.11	9.00 ± 0.23[c]

[a] Sample is defined in "Materials and Methods" (7.5 cm^2 surface area).

[b] Mean ± SE.

[c] Significantly different by Student's *t* test from corresponding controls ($p<0.05$).

crometer calibrated for 0.1 mm at 100 × and 430 ×. The mean thickness of the squamous epithelial layer (distance from epithelial surface through the basal layer of cells) was determined for each section. Then, using the micrometer to localize 0.1-mm segments through the thickness of epithelium (measurements parallel to the surface of tissue), cell counts were made within numerous random areas of defined dimensions (mean thickness × 0.1 mm). The number of cells composing the surface epithelium of control and infected skin at various intervals during the experiment was calculated from these data and expressed in terms of the previously described constant sample size.

Tissue fractionation and chemical procedures. Epithelium was separated into acid-soluble, lipid, nucleic acid, and protein fractions by a procedure modified for Schneider (10). Pooled epithelial preparations were homogenized at 4°C in 5% perchloric acid and centrifuged at 1000*g* for 15 min. The precipitates were washed with 80% and 95% ethanol and extracted twice at 56°C for 20 min each in diethyl ether:ethanol (1:3) for the removal of lipids. After centrifugation, the sedimented material was extracted with 5% trichloroacetic acid for 20 min at 100°C and analyzed for DNA (11). Following removal of nucleic acid, sediments were washed with acetone and diethyl ether, dried at 100°C overnight, and weighed in tared containers. In order to relate dry weight determinations to protein content, selected preparations were analyzed by the biuret procedure of Robinson and Hogden (12) as modified by Moore and Randall (13). Sediments were dissolved in 3% sodium hydroxide, and colorimetric and dry weight assays varied by a factor of less than 10%. The dry weight of lipid- and nucleic acid-free precipitates was therefore taken to indicate protein.

Results. Quantitation of hyperplasia. Examination of sequential sections of infected skin confirmed the well-documented morphological development of the hyperplastic lesion of fowlpox. As previously shown (1–3,6), the cutaneous infection involved a proliferative response with prominent hyperplasia of both epidermal and follicular epithelium with the presence of conspicuous intracytoplasmic viral inclusions.

Sections of control and infected skin were examined for quantitative increase in thickness and cellularity of surface epithelium as previously described. Results are shown in Table I. No significant thickening or hyperplasia was evident in the skin of control animals during the experiment. In contrast, infected skin exhibited marked hyperplasia of epithelium. Cellular proliferation with concomitant thickening of infected epithelium were first manifest as a significant increase

over controls at 48 hours after inoculation. Cellularity progressively increased to a maximum over the control 72 hours after infection.

We conclude from these observations that fowlpox virus induces the proliferation of cutaneous epithelium initially evident at approximately 48 hours after inoculation and culminating in a 2.5-fold increase in cell number at 72 hours.

Viral growth kinetics. Epithelium was obtained from infected chicks at 2-hour intervals for the first 24 hours after inoculation, at subsequent 12-hour intervals through 108 hours, and lastly, at 132 hours. Each preparation was homogenized in 2 ml of 0.06 *M* phosphate buffer, pH 7.6, serially diluted in buffer, applied to plucked scalps of a second series of animals, and the titer determined after 6 days.

Figure 1 illustrates viral growth kinetics of the *in vivo* infection. Consistent with previous electron microscopic observations (6), replication was associated with a latent period of approximately 22–24 hours and was exponential through 96 hours of infection.

Sequential increase of protein and DNA during infection. Epithelial preparations from groups of control and infected animals, collected at selected intervals, were pooled, fractionated, and analyzed for protein and DNA content as previously described. The results are given in Table II. Total protein content was first significantly elevated in infected tissue at 48 hours after inoculation and then progressively increased during infection to reach approximately 7 times the control value after 96 hours. In contrast to protein, DNA content was significantly elevated in in-

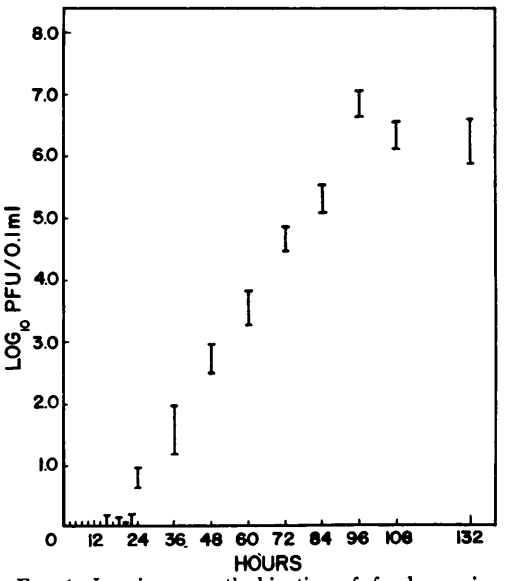

FIG. 1. *In vivo* growth kinetics of fowlpox virus in chick epithelium. Bars represent 0.95 fiducial intervals of mean titers. The infectivity of epithelial preparations from animals infected for less than 24 hours was not reproducible and in all cases represented a titer of less than 1 pfu/0.1 ml. These levels of infectivity are insignificant and were taken not to represent viral replication, but rather probably resulted from residual virus of the inocula.

TABLE II. Sequential Increase of Total Protein and DNA in Chick Epithelium Infected *in Vivo* with Fowlpox Virus.

Postinoculation (hours)	Protein (mg/sample[a])		DNA (μg/sample)	
	Control	Infected	Control	Infected
0	0.68 ± 0.15[b]	0.53 ± 0.08	8.85 ± 0.53	5.93 ± 0.83
12	1.20 ± 0.23	0.60 ± 0.08	9.53 ± 1.20	7.20 ± 0.83
24	1.20 ± 0.08	0.83 ± 0.15	9.60 ± 0.30	14.03 ± 1.28
36	0.90 ± 0.23	1.58 ± 0.15	6.15 ± 1.20	31.35 ± 4.73[c]
48	1.13 ± 0.15	2.33 ± 0.38[c]	10.05 ± 2.48	38.78 ± 2.63[c]
60	1.65 ± 0.30	3.23 ± 0.15[c]	13.57 ± 2.25	43.13 ± 0.75[c]
72	1.13 ± 0.23	4.13 ± 0.23[c]	13.28 ± 3.00	46.43 ± 5.56[c]
84	0.90 ± 0.23	4.58 ± 0.60[c]	10.88 ± 1.05	52.43 ± 4.20[c]
96	1.05 ± 0.08	7.28 ± 0.60[c]	16.50 ± 2.78	76.13 ±11.18[c]

[a] Sample is defined in "Materials and Methods" (7.5 cm^2 surface area).

[b] Mean ± SE (96-hour control data from 2 experiments; all other data from 3 experiments).

[c] Significantly different by Student's *t* test from corresponding controls ($p<0.05$).

fected epithelium after 36 hours and, like protein, was sequentially increased during infection to 7 times the control at 96 hours postinoculation.

Discussion. It is evident from comparison of the data of Table I and Fig. 1 that the initiation of viral replication preceeds a detectable hyperplastic response by at least 12 hours, and the titer increases past the period of cellular proliferation by approximately 24 hours. Although viral replication is initiated prior to induction of the proliferative response and subsequently is concomitant with hyperplasia, production of infectious virus is limited during this period to only 1% of the maximum titer. Thus, while all discernible evidence of cellular proliferation is confined to the period of infection between 48 and 72 hours postinoculation, 99% of the total virus titer is attained between 72 and 96 hours.

These observations indicate that the pathogenesis of the mature fowlpox lesion involves two almost distinct biological phases. An initial phase, predominant during the first 72 hours of infection, is characterized by initiation of viral replication and host tissue hyperplasia with concomitant production of a very small percentage of the total virus. In contrast a latter phase between 72 and 96 hours postinoculation marks the cessation of cellular proliferation with production of almost all infectious virus. Of basic biochemical significance to this apparent biphasic development of the fowlpox lesion is the relationship of viral replication and the induction of host tissue hyperplasia to the kinetics of DNA synthesis. Swallen (14) has demonstrated by autoradiography that chick epidermal cells infected *in vivo* show a higher percentage of labeled nuclei as compared to controls, suggesting that infection is associated with an increased incidence of intranuclear DNA synthesis. It is important to analyze the progressive increase in total DNA content of infected epithelium found in the present studies (Table II) in terms of synthesis of viral and cellular DNA. Toward this end experiments employing methylated albumin chromatography for the separation of viral and cellular DNA from infected tissue are currently in progress to investigate the time-course, sequential rate, and specificity of utilization of isotopic precursors.

Summary. Viral and cellular growth and sequential content of total protein and DNA were studied in chick epithelium infected *in vivo* with fowlpox virus. The first evidence of epithelial hyperplasia associated so prominently with fowlpox was noted after 48 hours of infection and was manifest as a 2.5-fold increase in cell number at 72 hours postinoculation. The initiation of viral replication preceded hyperplasia by at least 12 hours, and the titer increased exponentiallly through 96 hours of infection. Development of the mature lesion was accompanied by 7-fold increases in protein and DNA content of infected epithelium. Although viral replication was initiated prior to induction of the proliferative response and was subsequently concomitant with hyperplasia, production of infectious virus was limited during this period to only 1% of the maximum titer. Thus, while all discernible evidence of cellular proliferation was confined to the period of infection between 48 and 72 hours postinoculation, 99% of the total virus was attained between 72 and 96 hours. It is proposed that the pathogenesis of the *in vivo* lesion of fowlpox involves two almost distinct biological phases consisting of initiation of viral replicaton and epithelial hyperplasia with concomitant production of a very small percentage of the total virus during the first 72 hours of infection, followed by cessation of cellular proliferation with production of almost all infectious virus.

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Sex Specific Diuresis (32701)

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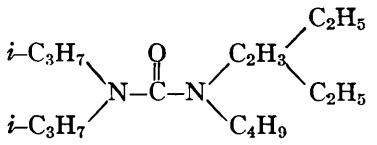
During a general biological investigation of a series of aminoalkylureas, certain of these compounds exhibited significant diuretic properties. The most potent of these, *N,N*-diisopropyl-*N'*-*n*-butyl-*N'*-diethylaminoethylurea (P-275),



was found to be an effective saluretic only in male animals. A close homologue *N,N*-diisopropyl-*N'*-isoamyl-*N'*-diethylaminoethylurea (P-286), differing from P-275 merely by a methyl group, has received extensive attention as a selective autonomic blocking agent (1–3). Whereas P-275 also has this blocking property, but is slightly less potent, laboratory study indicated that P-286 was devoid of diuretic activity. This was of interest in that the latter compound was found to be diuretic in the human and will be discussed in terms of the animal laboratory findings.

Materials and Methods. Evaluation of diuretic efficacy was determined mainly in conscious animals of two species. Male rats, weighing 50–100 gm, were subjected to the experimental procedure described by Lipschitz *et al.* (4) using a four-point biological assay. (5) Compounds were administered in the hydration fluid (isotonic NaCl, 25 ml/kg by stomach tube) and urine collected for 5 hours immediately thereafter.

Dogs of either sex were given test compound or placebo, orally in capsule form, and 60–90 min later were loosely restrained in a recumbent position. The bladder was emptied, employing an indwelling catheter. An iv infusion of 5% glucose was started and continued at a rate of 2 ml/min during the subsequent 2 hours of urine collection. All animals were without food for 16 hours prior to the experiment. Urinary Na^+ was determined in all experiments, and urinary K^+ in a few, using a Beckman DU flame spectrophotometer.

Certain surgical and hormonal treatments were completed, to investigate the sex-dependent diuretic effect of P-275. Female dogs were spayed under asceptic conditions and the animals were allowed a 30-day recuperative period before drug testing was continued. Male dogs treated with estrogen received 15 μgm/kg of estradiol benzoate daily im (9) for 8 days before drug testing was continued. Female dogs treated with testosterone received daily im injections of testosterone propionate in a dose of 0.5 mg/kg (10,11). Drug testing was continued beginning with the fourth week of such treatment. All injections of these hormones were made into the hind legs. The P-275 and P-286 were used as the HCl salts.

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