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Behavior of fowl, pigeon and canary pox viruses in chicks following intravenous inoculation.

by Anton Mayr.

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The skin is a preferred organ of manifestation in pox diseases. Epithelial cells offer particularly favorable conditions for multiplication of pox viruses. For this reason cutaneous injection is the method of choice in animal tests of viruses belonging to the pox group. It is used for such diverse purposes as initial virus culture, virus titration, production of vaccines, etc. The susceptibility of epithelial cells is so marked that even heterologous pox pathogens may be grown on the skin of a host. Most transmission and adaptation tests proceed from this premise.

Cutaneous injection is less suitable when virus strains are to be tested for their ability to produce generalized pox disease, as indicated, for example, in safety inspections of fowl pox vaccines. Cutaneous injection disrupts the pathogenetic chain of events by circumventing the natural mode of infection, which frequently prevents the development of maximal generalized pox disease despite inoculation of virulent virus. A mild form of the disorder may result, but frequently a local eruption of pustules at the site of injection is the only outcome, without subsequent generalization of the virus throughout the organism. In early days this method of producing a mild clinical course with an inherently virulent virus was called "variolation" or "ovination" and was used to vaccinate humans and sheep.

A generalized pox disease developing in the course of natural infection is a cyclic infectious disorder. Eruption of pustules on the skin and mucous membrane is preceded by a viremic stage in which the virus reaches its organs of manifestation via the hemal route. Viremia therefore represents a necessary intermediary link to generalized infection. It may be graduated artificially at any time by intravenous inoculation.

We have studied the behavior of fowl, pigeon and canary pox viruses in chicks after intravenous injection concurrently with our investigation of fowl pox vaccines. We pursued several goals in this connection.

For one, we searched for a simple test for safety determinations of fowl pox vaccines. In addition, we planned to explore the possibility of establishing a qualitative measure of the capacity of different fowl pox strains to produce generalized pox disease. Finally, the behavior of pigeon and canary pox viruses upon intravenous injection in chicks was to be compared with that shown by fowl pox strains.

Material and methods.

In order to obtain a scale of reaction potential of maximal breadth, we used strains of the most diversified origin and virulence. We apposed highly virulent strains to extremely weak ones, some of which are on the market as fowl pox vaccines due to their low virulence for the chicken.

We used 5 strains of fowl pox virus of varying virulence, designated HP-1, HP-2, HP-3, HP-4 and HP-5. HP-1 and HP-4 are highly virulent strains which regularly lead to generalized pox disease in chicks and hens, regardless of the mode of injection. They differ in other biological properties (1). Strain HP-1 was furnished by Dr. Gerriets. Strains HP-2, HP-3 and HP-5 are weakly virulent fowl pox strains (H-strains). They are available commercially as fowl pox vaccines on the basis of H-strains. Their cutaneous inoculation ordinarily produces only local pustules. No generalization takes place. We similarly had 5 different strains of pigeon pox viruses at our disposal. We designated these as TP-1, TP-2, TP-3, TP-4 and TP-5. The strains showed different behavior in cultures of chick embryo fibroblasts (1). They also show strongly dissimilar behavior in the chorioallantoic membrane (CAM) of the incubated chicken egg and in other biological properties. Strain TP-5 was particularly characterized. Its multiplication on the CAM produced substances that had a toxic effect on chick embryo fibroblast cultures (1). All strains produced local pustules in the chicken.

Prof. Dr. Hersberg furnished a strain of canary pox virus. His designation is KP-1. The virus is easily grown on the CAM of incubated chicken eggs and on chick embryo fibroblast cultures, where a visible cytopathological effect is produced.

The inoculum for chick tests consited of infected chorioallantoic membranes. Membranes were harvested at the peak of infection and stored at -20°C. They were treated 1:5 with m/90 phosphate buffer (pH 7.3), ground with sand and centrifuged for 20 minutes at 3000 RPM. The supernatant fluid was used in the preparation of appropriate virus dilutions.

We injected culture virus in some of the tests. This invariably involved virus grown in cultures of chick embryo fibroblasts according to a method described by us previously (1). At the peak of cytopathogenic effect the intracellular virus was liberated by agitation with glass beads or freezing and thawing between -60°C and +20°C, after

which the culture medium was isolated. Cellular debris was removed by spinning at low rotation. The virus was stored at -20°C until tests were initiated. The cultures contained pure, filtered bovine amniotic fluid (BAF) as medium for infection. In order to stabilize the virus during storage, we added 1% Molico in some tests. The preparation is a fat-free dietary milk in powder form made by Nestle, Lindau/Bodensee. We used a 10% solution of distilled water for sterilization. Sterilization took place in a steam vessel for 60 minutes. Admixture of Molico was tolerated without reaction by the chicks upon intravenous inoculation.

Inoculation of one-day chicks proceeded through the superficial, medial vein of the leg. 0.5 to 1.0 ml was injected per animal. Shortly before injection the chick's leg is immersed in a water bath of 52-54°C until the heat becomes unbearable for the operator's hand. The cutaneous vein now projects far enough to allow positive injection. An assistant must immobilize the chick on the table's edge. The position of the holding fingers is evident from Fig. 1. Injection must proceed very slowly. This is particularly important for injection of CAM homogenates. Overly rapid inoculation leads to lethal shock within a few minutes. The concentration of the inoculum is decisive. In the case of a 1:50 dilution of CAM, safe injection takes 5 minutes if 0.5 ml are introduced. Culture material, on the other hand, is harmless. It may be inoculated undiluted without detriment to the chick. The point of puncture is daubed with iodine after injection. A simple cotton bandage is applied if profuse hemorrhage should occur.

In the case of older chicks and half-grown hens we used the wing vein for intravenous injection.

In some parallel tests the inoculum was instilled intracerebrally. The customary method was used.

The animals were held in isolated buildings in separate boxes. The chicks were observed daily and examined for signs of generalized pox disease. Special attention must be paid to the beak, nose, eyes, comb and the extremities. These primary lesions were eliminated from the evaluation of generalization.

Virus titrations were carried out either on the CAM of 10day chick embryos (egg-ID_{50}), in tube cultures of trypsinized chick embryo fibroblasts (CID_{50}), or comparatively in both systems. Unless otherwise indicated, all titrations are applied to 0.1 ml. The technique of both types of titration has been described in earlier papers.

Tests and results.

In the basic test we injected all strains in the form of ground, infected chorioallantoic membranes intravenouly into 20 one-day chicks in each case. The course of infection was observed. The amount of

inoculum was 0.5 ml per animal, the titer was adjusted to $10^{-4.0}$. The tests were repeated several times in order to obtain significant results. The arrangement of individual animal groups was based on the principle of "random distribution." White Leghorn or partridge-colored Italians were employed. No variations in susceptibility were observed among the two breeds.

The individual virus types as well as the different strains showed very dissimilar behavior in 1-day chicks upon intravenous injection. Only the fowl pox strains were able to induce generalized pox disease. Pigeon and canary pox viruses failed to lead to generalized pox with eruption of pustules on the skin and mucous membrane. This action of the three virus types permits reliable determination and differentiation of fowl pox strains from pigeon and canary pox viruses.

The fowl pox strains (H-strains) also revealed marked variation with respect to their action on chicks. The variations involved 1. the percentage of capacity for generalization, 2. the incubation time and 3. the type and severity of generalized pox disease. Strain HP-1 invariably generalized 100%. The shortest incubation time was 4-5 days. A mixed skin and mucous membrane form developed, marked by maximal severity and high mortality. HP-4 was closest to HP-1. Its incubation time was doubled, however. This strain was characterized by large, wide and raised wart-like to cauliflower-like foci of pustules which healed but slowly. Essentially strain HP-4 led merely to a cutaneous form with low mortality. The other three H-strains, available commercially as pox vaccines, were far weaker in virulence. It is significant that they also produced generalized eruption on the skin of a certain number of chicks, but showed considerable variance in virulence. HP-2 produced 30% generalization, HP-3 60% and HP-5 even 90%. Strain HP-5 had the shortest incubation time with 8-12 days. The course of generalized pox disease induced by all three strains was milder than that produced by strains HP-1 and HP-4. Usually only cutaneous pustules developed, which dried and separated rapidly. The general condition of the animals frequently experienced only a slight disturbance. There were no fatalities.

Table 1 offers a compilation of these tests.

The ability to generalize shown by H-strain vaccines upon intravenous application was wholly unexpected. We therefore tested the dependence of generalization on the manner of instillation. Ground chorioallantoic membranes were employed once again, with titers adjusted to a uniform 10^{-4}. Results are reflected in Table 2.

Natural strains generalized in connection with every mode of infection, albeit in varying degrees. The immunogenic strains did not generalize after cutaneous and intramuscular infection. Their marked ability to generalize upon intracerebral injection was impressive, however. It is not very far below the level produced by intravenous application, considering that 0.5 ml was injected i.v. and only 0.05 ml intracerebrally.

We tested the quantitative conditions connected with generalized pox disease after intravenous infection by instilling graduated amounts of virus. Strain HP-1 of the 107th CAM passage served as model virus. The membrane suspensions were titrated for comparison on the CAM of the incubated chicken egg and in chick embryo fibroblast cultures by the tube method (1). We dispensed 1.0 ml intravenously in parallel titrations and 0.1 ml per egg and culture, in order to obtain better comparative values.

Results are shown in Table 3.

The tendency to generalize in the chick following intravenous infection was relatively high. The titer of the inoculum in the chick embryo, evaluated on the CAM, was $10^{-5.8}$ egg-ID_{50} and $10^{-6.0}$ CID_{50} in chick embryo fibroblast cultures, applied to 0.1 ml. The generalization titer in the chick was $10^{-4.33}$, applied to 1.0 ml.

When intravenous injection of chicks with CAM homogenates proceeds too rapidly, lethal shock could set in. Although the danger of shock diminishes with dilution, it nevertheless impeded inoculation. We therefore tested the behavior of culture virus after intravenous injection. The culture medium from which the virus is harvested consists of pure bovine amniotic fluid. It was safe for chicks. With respect to generalization, culture virus (chick embryo fibroblast culture) did not differ from egg virus during 45 passages. We studied one strain of fowl, pigeon and canary pox viruses.

Results are compiled in Table 4.

Fowl pox strain HP-1 generalized after 45 culture passages as before. Pigeon and canary pox viruses again failed to generalize.

Finally we investigated the action of the various H-strains upon intravenous injection in older chicks. We employed chicks 2, 6 and 8 weeks old. The inoculum consisted of ground CAM or culture virus with nearly identical titers. Results are shown in Table 5.

We did not observe a difference in percentage of generalization in H-strains when instilled in older chicks. The percentage approximated that of 1-day chicks. The course of disease in older chicks frequently was less severe than in 1-day chicks, however. This was expressed in a less crowded pustule eruption, faster drying and eschar formation on the exanthema, and in less severe involvement of the animals' general health.

Discussion of results.

When chickens or chicks are inoculated cutaneously with weakly virulent fowl pox strains or with pigeon or canary pox viruses, primary pustules develop at the site of injection. A generalized disease does not occur. Aside from low-grade differences in local formation of pustular foci, all three types of virus show the same reaction. Cutaneous inoculation masks decisive differences in virulence that exist not only between the three forms, but also among individual strains.

Our studies demonstrate that intravenous injection of 1-day chicks permits precise determination of differences in virulence between the various virus strains. This furnishes a method which, for one, allows differentiation of questionable fowl and pigeon viruses and, more important, exact analysis of fowl pox strains relative to their virulence for the chicken.

In the production and inspection of fowl pox vaccines, intravenous inoculation of chicks may be employed for two practical purposes: For the testing of safety and for the determination of a vaccine's efficacy.

Current fowl pox vaccines available on the world markets are based either on weakly virulent fowl pox (H) or pigeon pox (T) strains. T-strains heterologous for the chicken were unable to evoke generalized pox disease in our chicks. H-strains behaved differently. They invariably led to a general eruption of pustules in a certain percentage of chicks, i.e., they were considerably more dangerous than the T-strains in regard to safety and reliability of vaccines. It is significant, however, that their virulence for chicks was variable. Variations involved 1. The percentage of generalization, 2. the incubation time and 3. the type and severity of generalized pox disease. Thus, for example, one H-strain invariably generalized only 30% of the chicks with an incubation time of 10-12 days, whereas another vaccinal strain generalized 90% and showed a considerably shorter incubation time.

The most important step in the production of a fowl pox vaccine is the correct selection of strains. Intravenous injection of chicks now furnishes a simple safety test. Although we found considerable variations in virulence among H-strains, we have not discovered one that fails to produce generalized pox disease. The search must continue. It is certain that all immunogenic strains which lend themselves to artificial inducement of generalized pox in the chick and adult hen are dangerous epidemiologically.

It is particularly noteworthy in this connection that vaccinal strains always caused a certain percentage of generalization upon intracerebral injection. This behavior underscores their danger. Perhaps H-strains can be found which at least do not generalize intracerebrally. For this reason intravenous determinations of virulence should be augmented by a test for intracerebral reaction.

Tests of the efficacy of a fowl pox vaccine were usually carried out by cutaneous reinfection and evaluation of the resultant reaction, supplemented in some cases by determination of virus-neutralizing antibodies (2). We are engaged in applying intravenous injection of chicks to determinations of efficacy. Graduated doses of virulent strain HP-1 regularly produce generalized pox disease in half-grown hens, which may then be used as an indication of the vaccines' effectiveness. The result of such a test furnishes more information about the actual protective effect of a vaccine than cutaneous reinfection. Since the range of virus dosages required for generalization is relatively wide, it is possible to measure a vaccine's efficacy. Culture virus is an excellent test virus. There is no danger of shock for the chick, even in its undiluted form, and it may be stored without loss of infectivity upon addition of 1% Molico (fat-free dietary milk in powder form) either at -20°C or after lyophilization.

Illustration

Fig. 1. Intravenous inoculation of one-day chicks.

Tables

Table 1. Variable behavior of different fowl, pigeon and canary pox virus strains in one-day chicks after intravenous injection. (Titer of inoculum $\sim 10^{-4.0}$ egg-ID_{50})

Virus	Strain		Behavior after i.v. injection: % of generalization	Incubation time	Type and severity of disease
Fowl pox	Strongly virulent natural strains	HP-1	100	4-5 days	Skin & mucous membrane, severe course, 40% mortality
		HP-4	90	8-10 days	Skin, severe course, slow restitution
	weakly virulent vaccinal strains	HP-2	30	10-12 days	Skin, mild course, rapid restitution
		HP-3	60	8-10 days	Skin, mild course, rapid restitution
		HP-5	90	5-7 days	Skin, mild course, rapid restitution
Pigeon pox	vaccinal strains	TP-1	0	-	-
		TP-2	0	-	-
		TP-3	0	-	-
		TP-4	0	-	-
		TP-5	0	-	-
Canary pox	virulent strain	KP-1	0	-	-

Table 2. Dependence of generalization on the mode of virus application, using different fowl pox strains.
(Titer of inoculum $\sim 10^{-4}$ egg-ID_{50})

Strain	% of generalization after 1. cutaneous injection	2. intramuscular injection	3. intracerebral injection	4. i.v. injection
HP-1	100	100	100	100
HP-2	0	0	30	30
HP-3	0	0	40	60
HP-4	20	20	80	90
HP-5	0	0	60	90

1. 0.1 ml rubbed into feather follicles.
2. 0.5 ml injected.
3. 0.05 ml injected.
4. 0.5 ml injected.

Table 3. Dependence of generalization after intravenous inoculation of chicks upon the amount of virus, strain HP-1 (107th CAM passage)

Dilution of inoculum	Result of titration chick (1.0 ml)	egg (0.1 ml)	culture (0.1 ml)
10^{-2}	12/12	8/8	8/8
10^{-3}	12/12	8/8	8/8
10^{-4}	8/12	8/8	8/8
10^{-5}	3/12	8/8	8/8
10^{-6}	0/12	3/8	4/8
10^{-7}	0/12	1/8	0/8

Chicks: The denominator indicates the number of animals infected, the numerator gives the number of animals with generalized disease.

Eggs: The denominator indicates the number of eggs infected, the numerator gives the number of eggs showing virus-specific lesions of their CAM.

Cultures: The denominator indicates the number of culture tubes (chick embryo fibroblast cultures) infected, the numerator gives the number that showed virus-specific changes.

Table 4. Behavior of culture virus (chick embryo fibroblasts) upon intravenous injection of chicks.

Virus	Strain	19th culture passage Titer (*)	19th culture passage Generalization (**)	45th culture passage Titer (*)	45th culture passage Generalization (**)
Fowl pox	HP-1	5.0	20/20	5.3	20/20
Pigeon pox	TP-2	5.5	0/20	4.6	0/20
Canary pox	KP-1	4.6	0/20	6.0	0/20

(*) $-\log_{50}$ CID_{50}/0.1 ml

(**) the denominator indicates the number of animals infected, the numerator gives the number of animals with generalized disease.

Table 5. Behavior of H-strains after intravenous inoculation of older chicks. (Titer of inoculum $\sim 10^{-4.0}$)

Strain	% of generalization in chicks 2 weeks old	6 weeks old	8 weeks old
HP-1	100	100	100
HP-2	30	30	35
HP-3	60	50	60
HP-4	90	90	100
HP-5	85	95	90

Literature

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