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CHEMOTHERAPEUTIC STUDIES ON THE VIRUSES OF SHEEP ABORTION AND MOUSE PNEUMONITIS (NIGG) WITH SPECIAL REFERENCE TO AN EMBRYONATED EGG METHOD

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As part of a programme of research into the chemotherapy of virus infections, the need arose for a simple preliminary test which would select from numerous compounds, often available in only small amounts, those which might be of value in the chemotherapy of virus disease. The use of experimental virus infections in embryonated eggs is now widely accepted for such purposes and several techniques have been described. The aim of this work was to develop one such technique and to establish its value by using compounds known to affect virus proliferation—a condition which restricted investigation to viruses of the psittacosis-lymphogranuloma group (Findlay, 1951).

In investigating this group of viruses in embryonated eggs most workers have grown the virus in the yolk sac, into which they have also introduced the compound under investigation, and have measured antiviral action in terms of the extended life of the embryo. The practical disadvantages of this method are that frequent inspection of the embryo is necessary to determine the time of death, and that death must be demonstrated as being due specifically to virus multiplication. This latter usually involves the staining and examination of yolk sac smears—a somewhat tedious process.

These disadvantages could be overcome if a virus typical of the psittacosis-lymphogranuloma group, which would produce well-defined lesions susceptible of visual assessment, could be grown on the chorioallantoic membrane, the compound being given by some alternative route. For reasons of convenience the choice of virus was directed to those not pathogenic to man. Earlier attempts to establish mouse pneumonitis (Nigg) virus, by serial passage on the chorioallantoic membrane of virus from mouse lung, were discouraging. Learning of this difficulty, Mr. W. M. Brownlee, a veterinary colleague, aware of the discovery of a virus associated with ewe abortion (Stamp, McEwen, Watt, and Nisbet, 1950), drew attention to its possible suitability and obtained pathological specimens from a local case of the disease. The virus isolated from this material produced well-defined lesions on the chorioallantoic membrane and so was used to establish the test described. The use of this organism has been previously described (Dickinson and Inkley, 1951).

Results obtained from the embryonated egg test have been compared with results obtained by Dr. Dickinson using Nigg virus in mice. This has made possible some quantitative assessment of the embryonated egg test. It is assumed that the use of mice in chemotherapeutic studies is generally accepted as providing information relevant to disease in human beings. Details of the mouse-tests are given in the Appendix.

MATERIALS AND METHODS

Abortion Virus

Primary Isolation of the Virus of Sheep Abortion.—Smears of the foetal membranes, stained by Machiavello's stain, contained numerous elementary bodies, particularly in the region of the cotyledons. Portions of the cotyledons were ground in lemco broth to give an approximately 10% tissue suspension and centrifuged at 1,000 r.p.m. for 10 min. Streptomycin was added to the supernatant fluid to make a final concentration of 1,000 u./ml.; penicillin was avoided, since at this stage it was felt that the organism might be penicillin sensitive.

The inoculum thus prepared was placed on to the chorioallantoic membranes of 10-day embryos (prepared by the dropped-membrane technique); after a further 7-day incubation at 37° the membranes

were covered with discrete small lesions (Fig. 1). An inoculum for a second passage was made by grinding one of these infected membranes in 5 ml. lemco broth, centrifuging the suspension so prepared at 1,000 r.p.m. for 10 min., and then filtering the supernatant fluid through a gradocol membrane of pore size 1 μ; lesions were produced by dilutions of 1/10 and 1/100 of this filtrate.

Properties of Abortion Virus.—These are described by Stamp *et al.* (1950); in addition the following properties commend its use as a test organism.

(1) *Culture.*—After 20 passages on chorioallantoic membranes there was no apparent alteration in the lesions induced by the virus. Extremely high yields of virus were produced by yolk sac inoculation.

(2) *Freeze Drying.*—Virus suspensions, prepared by grinding infected chorioallantoic membranes or infected yolk sacs in broth, were successfully freeze-dried; the yolk sac suspensions proved particularly effective in maintaining the viability of the virus.

(3) *Viability.*—Infected whole chorioallantoic membranes suffered little loss of viable virus when kept at −20° or −70° for several months. Suspensions of infected yolk sac material in broth remained viable for months at −70° and were valuable as stock inocula for test purposes.

(4) *Response of Host.*—Uniform lesion patterns on the chorioallantoic membrane were consistently formed and were readily assessed visually.

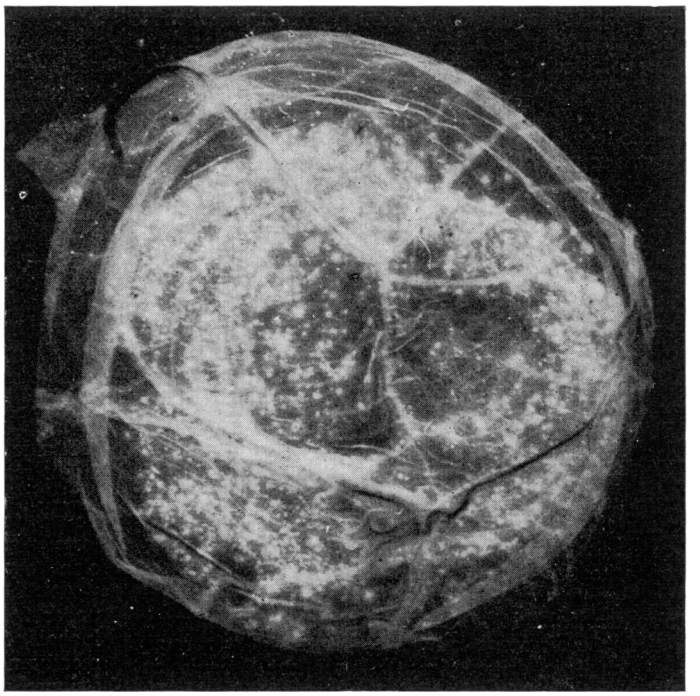

FIG. 1.—Lesions produced by sheep abortion virus on the chorioallantoic membrane (7 days' incubation). Magnification ×2.

Nigg Virus

Well-defined lesions on the chorioallantoic membrane were eventually obtained by the following procedure. Yolk sacs of 10-day embryos were infected with Nigg virus, obtained by gradocol filtration (pore size 1 μ) of a suspension of infected mouse lung. On death, each yolk sac was ground with 10 ml. broth and centrifuged at 2,000 r.p.m. for 10 min. The virus-rich supernatant material was collected, the fatty layer being avoided, and this provided a suitable inoculum when diluted 1/10 in lemco broth. These yolk sac inocula, unlike those of abortion virus, were relatively labile and did not produce the desired lesion pattern when inoculated directly on to the chorioallantoic membrane after storing for 2–3 weeks at −70°; they kept sufficiently well, however, to provide seed for yolk sac infection when fresh material was required (approximately one year at −70°). Their viability was maintained on freeze drying.

Route for Drug-inoculation

Since the virus was to be grown on the chorioallantoic membrane, only the route for drug-inoculation had to be decided. For practical reasons it had to be either the yolk sac or the allantoic sac. To compare the two routes the antibiotics aureomycin, chloramphenicol, and oxytetracycline ("terramycin") were used.

11-day embryos were given 0.2 ml. of aqueous two-fold dilutions of the drugs by one or other route. Abortion virus was placed on to the dropped chorioallantoic membrane 2–3 hr. later. Groups of six embryos were used at each dose level. Incubation was continued for a further 7 days. Distinct end points were obtained.

TABLE I

RELATIVE EFFICACIES OF DRUGS BY DIFFERENT ROUTES OF ADMINISTRATION

Drug	Inhibitory Dose* in mg./Egg	
	When Given by the Yolk Sac	When Given by the Allantoic Sac
Oxytetracycline ..	0·062	0·25
Aureomycin ..	0·25	1·0
Chloramphenicol ..	1·0	1·0

* Dose which prevents the formation of lesions on the chorioallantoic membrane. All compounds were toxic at 4·0 mg./egg.

As is shown in Table I, aureomycin and oxytetracycline are both more effective when given via the yolk sac. Some four times the amount of drug must be given into the allantoic sac to achieve the result obtained by yolk sac inoculation. With chloramphenicol, however, this does not hold, and both routes

are equally effective. Although no explanation is offered, the results are consistent with the known physiological functions of the allantoic sac and yolk sac.

The superior sensitivity of yolk sac inoculation made this the method of choice. Subsequently, when it was found that 200 u. each of penicillin and streptomycin given by the yolk sac did not affect abortion virus or Nigg virus, the various compounds under test were dissolved or suspended in an aqueous penicillin–streptomycin solution (1,000 u. of each/ml.). This practice is of great value, since many substances are difficult to sterilize and the yolk sac is particularly susceptible to infection by bacteria.

Method Finally Adopted

Embryonated egg techniques are described by Beveridge and Burnet (1946). For convenience, the modifications found useful are described in detail.

Embryonated eggs of 11 days' incubation are candled, and on each a position, just clear of the main allantoic vein, is marked on the shell, so as to be on the greatest diameter of the egg; diametrically opposite this a second mark is made to indicate the position for yolk sac inoculation; the air space is also marked. It is convenient to use only eggs in which the air space is at the "broad end" (normal). The shell is drilled for the yolk sac inoculation, sufficiently widely to permit the passage of a No. 12 hypodermic needle; the exposed shell membrane is clarified by one drop of hot wax (70°) which is then flattened to a very thin layer with a hot metal spatula. This latter procedure results in a sterile inoculation area and provides a good base for the subsequent wax seal. While awaiting introduction of the compound, this waxed area is kept downwards in order to orientate the yolk sac suitably. 0.2 ml. of a solution or suspension of the compound in water, containing 1,000 u./ml. each of penicillin and streptomycin, is then introduced into the yolk sac. For this the waxed area is brought uppermost and the needle thrust sharply 1 in. into the egg, which is transilluminated from the end opposite the air space. The yolk sac is displayed quite distinctly and inoculation can be seen to be successful. Occasionally, on withdrawing the needle, a small amount of allantoic fluid escapes but readily runs off the waxed area. The hole is then sealed with hot wax.

The egg, now containing the test compound, is drilled at the air space and a small triangle of shell is removed from the position marked near the allantoic vein. The chorioallantoic membrane is then "dropped" by wetting the exposed triangle of shell membrane with one drop of sterile broth and gently parting the fibres with a ball-ended glass rod. The chorioallantoic membrane usually falls away without further treatment. (Non-specific lesions resulting from this operation are extremely rare. The most common fault of the novice is the piercing of the chorioallantoic membrane, with the subsequent formation of a bubble of air in the allantoic cavity; in experienced hands this event is rare and about 100 eggs can be prepared in 20–30 min.) The virus inoculum is then deposited on to the "dropped" membrane, usually some 4–5 hr. after introducing the compound under test. The triangle of shell membrane is sealed with 3 drops of hot wax, pressed on with a warm spatula to give a perfect seal. In early work the inoculum was 0.2 ml. of a broth suspension prepared by grinding a fresh infected membrane in broth and centrifuging for 10 min. at 2,000 r.p.m., the supernatant fluid being made up to 20 ml. with broth. More recently, yolk sac suspensions, distributed in 1-ml. amounts and kept at −70°, have proved more convenient and are diluted up to 20–25 ml. in broth when required. Even distribution of the virus is ensured by gently raising in turn each side of the tray containing the sealed eggs.

The eggs are then incubated for a further 7 days at 37° and inspected daily to judge deaths resulting from the toxic effect of the various compounds; this latter is not really necessary unless detailed information on toxicity is required. Twofold dilutions of the compound, to include the dose toxic to the embryo, are tested; groups of six embryos at each level are sufficient to provide information.

At the end of the test period, the eggs are opened under 2% formalin by cutting the shell completely in half, the half containing the area of "dropped" membrane being retained. The membranes are stripped away from the shell and examined in petridishes. Lesions are readily obvious whilst stripping, and membranes bearing them can be discarded and the compound involved designated inactive. More careful scrutiny is applied to those with less distinct lesions. The embryos are not killed by the growth of the virus on the chorioallantoic membrane, and deaths can be related to the toxicity of the compound under investigation. It is not customary to examine the membranes from embryos which have died.

Results

Several compounds, previously described as active against viruses of the psittacosis-lymphogranuloma group under various conditions and in clinical use, have been examined by the embryonated egg test against both sheep abortion virus and Nigg virus. The results of the tests are summarized in Table II. Most of the figures were obtained from series in which twofold dilutions of drug were employed. It can be taken, where a figure is given as showing inhibition, that a dose of half that magnitude was inactive. A more exact measurement was not sought. Even so it is apparent that the order of effectiveness against both viruses is, on a weight basis, oxytetracycline> aureomycin>chloramphenicol. Both viruses appear to be equally sensitive to oxytetracycline and aureomycin, but Nigg virus seems rather more resistant to chloramphenicol than does the virus of sheep abortion.

TABLE II

EFFECT OF DRUGS ON VIRUS INFECTIONS IN EMBRYONATED EGGS AND IN MICE

Drug	Inhibitory Dose* (mg./Egg)		*Activity in Mice Against Nigg Virus (Drug given Subcutaneously from Day of Infection)†
	Abortion Virus	Nigg Virus	
Oxytetracycline ..	0·06	0·06	+++ (0·02 mg./g./day)
Aureomycin	0·25	0·25	+++ (0·02 mg./g./day)
Chloramphenicol ..	1·00	2·00	++ (0·02 mg./g./day)
Penicillin ..	Inactive 6·66 ≡10,000 u.	1·33 ≡2,000 u.	+ (0·13 mg./g./day) ≡4,000 u./mouse/day
Sulphathiazole	Inactive 14·7	14·7	+ (0·2 mg./g./day)
p-Aminobenzoic acid	Inactive 27·4	Inactive 27·4	0 (2·0 mg./g./day) orally

* Dose which prevents the formation of lesions on the chorioallantoic membrane.

† +, prolongation of life only; ++, no deaths, but lungs involved; +++, no deaths, lungs not involved.

Nigg virus is sensitive to penicillin and sulphathiazole. Abortion virus is not affected by penicillin and sulphathiazole at comparable concentrations: with penicillin, indeed, even five times the amount which inhibits Nigg virus is without effect. Neither virus is sensitive to *p*-aminobenzoic acid.

It is further evident from Table II that, for Nigg virus, the results given by the *in ovo* test closely follow those obtained from the mouse test. Details of the mouse test will be found in the Appendix and Table III.

Tests were also done in which the virus was established some time before the drug was introduced. For routine tests, however, this is undesirable, on account of the extra hazards inherent in the manipulation of infected eggs. Results from tests in which the infection had been established for as long as 24 hr. were similar to those in which the infection followed drug inoculation; but the mortality rate of embryos was very high, no doubt due to interference with respiration from the slowly absorbed liquid inoculum distributed around the chorioallantoic membrane.

DISCUSSION

In establishing the test described here for the examination of compounds for antiviral action, evidence has been obtained that *in ovo* tests are of value in chemotherapeutic studies of viral infections. The quantitative nature of the test is well demonstrated for Nigg virus, which may be considered a natural infection of mice. The disease, experimentally induced in mice by this virus, responds favourably to several of the compounds tested; the response of the *in ovo* infection to these same compounds is always of a like order. Several workers have studied other viruses on the chorioallantoic membrane, but none, so far as I am aware, were in a position to justify the method, since therapeutic agents active against the viruses they used were not available.

There is reason to suppose, in view of the physiological nature of the yolk sac, that a compound given into this organ is carried by the blood circulation to the embryo and then to the chorioallantoic membrane where it may be excreted into the allantoic sac. Thus the embryo itself experiences the toxic effects of the compound before it reaches the virus in the chorioallantoic membrane, so that the method affords a good measure of the therapeutic index of the drug—relative to the chick embryo. This may or may not be similar to the index obtained with a mammalian species. In contrast, when both compound and virus are introduced into the yolk sac the conditions favour the compound and, to this extent, such a test is more sensitive in determining the drug effect. The unfortunate result in practice is that compounds may appear to be active by *in ovo* tests and yet prove ineffective by animal tests, so that *in ovo* tests come to be held in disregard. It is against such a tendency that this paper is largely directed, since, in the present state of viral chemotherapy, the embryonated egg serves as the most powerful available tool in a field of investigation which must inevitably be somewhat empirical.

The use of abortion virus as a test organism in the search for chemotherapeutic agents against larger viruses suffers the disadvantages inherent in the use of any organism regarded as typical of a group. It would be better if attention could be directed to the particular disease organism for which therapy is sought. As a convenient experimental tool, however, the abortion virus possesses advantages, despite the fact that its sensitivity to chemotherapeutic agents is somewhat different from that of the Nigg virus.

SUMMARY

1. An embryonated egg test for the detection of antiviral agents is described. Its advantages and limitations are discussed.

2. Several compounds, known to affect the multiplication of large viruses, have been examined by this method against the viruses of sheep abortion and mouse pneumonitis (Nigg).

3. A comparison of the egg test and a mouse test (described in the Appendix) has been effected for Nigg virus, and a correlation which is markedly quantitative has been demonstrated.

4. The isolation of the strain of abortion virus used is described, and its advantages as a test organism set forth.

5. The value of embryonated egg tests in the present stage of viral chemotherapy is re-emphasized.

The author is indebted to Dr. Löis Dickinson for the primary isolation of the strain of abortion virus used in this work; for her keen interest in the development of this test; and for permission to quote the results of her mouse experiments, given in detail in the Appendix.

He is also indebted to Mr. W. M. Brownlee for his help in obtaining the pathological specimens; Dr. A. D. McEwen for helpful information on the abortion virus; Mr. G. Whiting for the photography; and the late Sir Jack Drummond, F.R.S., and Mr. C. E. Coulthard, who, by their keen interest, encouraged this work.

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APPENDIX

TESTS AGAINST MOUSE PNEUMONITIS (NIGG) VIRUS IN MICE

By Löis Dickinson

In vivo tests against mouse pneumonitis (Nigg) virus were carried out in order to compare the results of the *in ovo* test, described in the paper, with results obtained when the compounds were tested against the infection in mice produced by this organism.

Methods

0.05 ml. of a suspension of Nigg virus in lemco broth was given intranasally to groups of 20-g. albino mice under light anaesthesia. The stock virus consisted of infected lungs ground with 5 ml. broth per lung. This material was mixed with an equal volume of 10% glucose and freeze-dried in 1-ml. amounts. For tests, each ampoule was made up to 10 ml. and used undiluted, preliminary titrations indicating that this amount would kill most mice and infect all. All mice receiving 1/10th this dose showed infected lungs after 12 days, but only about 25% died (Table III). In every experiment the control group was the last group to be infected.

Treatment was commenced 1 hr. before infection, except where otherwise stated, and drugs were given twice daily, either subcutaneously or by stomach tube, for 12 days. Survivors were killed at 12 days and the lungs examined for evidence of infection. Lungs showing total consolidation were assessed at 4 (5 if death had occurred) and values of 3, 2, and 1 given for lesser consolidation. When the drugs had a pronounced effect on survival time, several mice were left on test for 10 days after the cessation of treatment to see whether the disease "flared up."

Table III

DRUG ACTION AGAINST NIGG VIRUS IN MICE

Drug	Dose/Day in mg./g.	Survivors at 12 Days	Survivors' Average Lung Assessment	Flare-up Test	
				Deaths	Infected Lungs of Survivors
Test 1	*(a) Normal Infecting Dose of Virus*				
Penicillin..	1,000 u./mouse (S.C.)	8/10	2·3		
Controls..		1/10	4·6		
	(b) High Infecting Dose of Virus				
Penicillin..	1,000 u./mouse (S.C.)	2/18	—		
Controls..		1/18	—		
Test 2					
Penicillin..	4,000 u./mouse (S.C.)	15/19	3·0	4/7	3/3
Sulphathiazole	0·4 (S.C.)	18/20	1·6	4/8	3/4
,,	0·2 ,,	16/20	0·5	—	—
p-Aminobenzoic acid	2·0 (oral)	3/10	3·5	—	—
Control..		6/20	3·4	—	—
Test 3					
Chloramphenicol	0·2 (oral)	11/11	—	0/11	3/11
,,	0·02 ,,	10/10	1·1	—	—
,,	0·02 (S.C.)	7/8	1·0	—	—
Aureomycin	0·1 (oral)	10/10	—	0/10	0/10
,,	0·02 ,,	10/10	0	—	—
,,	0·02 (S.C.)	9/9	0	—	—
Oxytetracycline	0·05 (oral)	10/10	—	0/10	0/10
,,	0·02 ,,	10/10	0	—	—
,,	0·02 (S.C.)	1 /10	0	—	—
,,	0·02 (neutral)	10/11	0	—	—
Controls..		4/12	3·0	—	—
Controls (1/10th infecting dose of virus)		8/12	2·1	—	—
Test 4	*Drugs for Four Oral Doses Starting One Day after Infection*				
Chloramphenicol	0·8 (total dose)	8/10	2·1	—	—
Aureomycin	0·4 ,, ,,	9/10	1·7	—	—
Oxytetracycline	0·2 ,, ,,	10/10	1·5	—	—
Controls..		4/12	3·0	—	—

Results

All results are given in Table III and indicate the efficacy of oxytetracycline ("terramycin"), aueromycin, and chloramphenicol in that order. All these drugs suppressed the development of lung lesions, but a higher dose of chloramphenicol was required. Both oxytetracycline and aureomycin were effective at 0.02 mg./g./day orally or subcutaneously, and prevented the development of the

disease for 10 days after treatment had ceased, at 0.05 and 0.1 mg./g./day respectively. Previous work had shown that 0.02 mg./g. aureomycin was not effective against a high infecting dose of the virus and 0.01 mg./g. did not protect mice significantly in the normal test.

When treatment was limited to four doses starting one day after infection, all three drugs still exerted some protective action. A total dose of 0.8 mg./g. choramphenicol was less effective than 0.4 mg./g. aureomycin or 0.2 mg./g. oxytetracycline; there were no deaths in the oxytetracycline group, but the lungs showed evidence of infection.

The results with penicillin indicate the necessity for giving the maximum dose if an effect is to be noted; the effect was not observed when 400 u./mouse was given, and even 1,000 u./mouse had no effect if the infecting dose of virus was high. This drug only delayed death and did not markedly suppress lung lesions.

Sulphathiazole suppressed lung lesions to some extent, but was not nearly so effective as the antibiotics. *p*-Aminobenzoic acid was inactive.