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PRELIMINARY OBSERVATIONS ON THE VALUE OF THE GUINEA-PIG IN DETERMINING THE INNOCUITY AND ANTIGENICITY OF NEUROTROPIC ATTENUATED HORSESICKNESS STRAINS

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Because horsesickness is enzootic over the greater portion of South Africa, and routine annual immunisation with live attenuated virus vaccine has been used as the chief means of control for many years, it is difficult to breed, and is becoming increasingly difficult to purchase, adequate numbers of susceptible horses for research purposes. Meanwhile the spread of the disease to extensive regions outside the continent of Africa has focussed attention upon the urgent necessity for further research into a number of aspects of the disease. However, the implementation of any research programme was hampered by the lack of large numbers of a readily available, inexpensive laboratory animal of uniform susceptibility or particular suitability for a specific purpose. Therefore, it was decided to reinvestigate the possibility of finding an experimental animal to supplement the mouse, which is in general use, but which has many limitations.

The original work on the attenuation of horsesickness virus by intracerebral passage as the basis of a method for the immunisation of horses and mules (Alexander & Du Toit, 1934) had shown that although attenuation appeared to be produced more rapidly in guinea-pigs than in mice, the mouse appeared to be more suitable for routine vaccine production and from that time no further use has been made of the guinea-pig for virus propagation or attenuation.

Similarly, after it had been shown that the intraperitoneal protection test as a screening method for the study of immunity in yellow fever, as developed by Sawyer & Lloyd (1931), could not be adapted to similar studies in horsesickness, because the percentage mortality following intraperitoneal injection of the particular strains of neurotropic adapted virus used was low and inconstant, the mouse intracerebral test was developed (Alexander, 1935). As a result the guinea-pig virtually disappeared from the research field in this disease. This is illustrated by the fact that McIntosh (1958), in investigating the difficulty experienced in isolating strains of wild virus from immunised horses, made use of the dog, whose susceptibility has been confirmed since the original observations of Theiler (1906, 1910), and the ferret, in addition to mice and used the rabbit for the production of type immune serum.

A perusal of the comparatively limited literature on horsesickness shows that considerable attention has been paid to the antigenic classification of virus strains. The position in this classification, after demonstrating an adequate degree of attenuation for horses, served as the basis for the selection of a particular strain for routine vaccine production, but no reason appears to have been advanced for the selection of strain A501, for example, in preference to any of the other thirteen strains in Group I.

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In addition, after it had been shown that even after 158 serial intracerebral passages in mice, Strain 449 (Group I) did not produce a fatal encephalitis after injection into the brain of a susceptible horse (Alexander, 1935) it appears to have been assumed that the neurotropic affinity of all strains for horses would not be increased to a dangerous level during attenuation by serial intracerebral mouse passage. In any event there is no record that other vaccine strains were tested by intracerebral injection into either horses, mules or donkeys. Recent events have shown that this has been a serious omission.

In the work which forms the basis of this report extensive use has been made of the guinea-pig and it has been shown that this laboratory animal is of great value for specific purposes.

Materials and Methods

The seven antigenically different strains, incorporated in the routine vaccine at the time, were used, viz. A501, OD, L, Vryheid, VH, 114 and Karen. The origin of these strains has been reported by McIntosh (1958). In addition there was available a further strain 7/60 designated as the prototype of a new Group 9 (Howell, 1962), at its 120th adult mouse passage level.

These strains are maintained as freeze dried preparations of 0·5 ml amounts of 10 per cent emulsions of infective mouse brain in buffered lactose peptone,* sealed in glass ampoules and stored at −20°C and in the dry ice cabinet.

For the preparation of guinea-pig inocula a single ampoule of each type virus was reconstituted in 5·0 ml of buffered lactose peptone and injected intracerebrally into each of a family of 4- to 6- day-old mice, the dose being 0·03 ml. Each family was housed separately in a metal box provided with a separate food hopper and water bottle. Within two to three days all the mice showed symptoms of encephalitis. Those *in extremis* were etherised, the brains removed with aseptic precautions and pools of each type stored intact separately in screw cap containers at −20°C. Frozen material was removed as required for emulsification and clarification by centrifugation in an angle centrifuge at 3,000 rpm for 15 minutes.

Neutralisation test

The technique was essentially that described by Alexander (1935). Serial five-fold dilutions of serum were prepared in buffered lactose peptone to produce final dilutions of 1/5 to 1/3125. The virus titre of the reconstituted antigens was determined by intracerebral inoculation of suckling mice with serial ten-fold dilutions and was used in a dilution calculated to contain between 20 and 100 LD_{50} per 0·03 ml. The serum virus mixtures were incubated in a water bath at 37°C for one hour and then injected immediately into not less than six mice for each dilution.

End points were calculated by the method of Reed & Muench (1937).

Laboratory animals

Mice were obtained from the Institute's colony, which originated from the Rockefeller Foundation yellow fever strain of Swiss albino mice and has been maintained as an in-bred closed colony for more than 30 years. Suckling mice four to six days old were used throughout.

* M/50 phosphate buffer pH 7·2–7·4, 1 per cent Difco peptone and 5 per cent lactose.

All guinea-pigs were obtained from the Institute's colony. The origin of this colony of white guinea-pigs is obscure, but it too has been maintained as a closed colony for at least 20 years. Young adult females were used throughout. Rectal temperatures were taken each morning before feeding, when the habitus of each animal was noted carefully. For virus isolation brain suspensions (2 per cent) and spleen suspensions (10 per cent) were prepared in buffered lactose peptone, and clarified by centrifugation for injection intracerebrally into mice.

Experimental Investigations

I. *The effect of the intraperitoneal injection of vaccine virus strains into guinea-pigs*

It was decided to produce complement fixing antisera against all the vaccine virus strains in guinea-pigs by repeated intraperitoneal injection. Quite fortuitously strains 114 (Group 6) and Karen (Group 7) were being used for other purposes

Table 1.—*Result of the intraperitoneal injection of seven vaccine virus strains into guinea-pigs*

Guinea-pig No.	Inoculum			Reaction†	Diagnosis	Virus reisolation	
	Type	Strain	Titre			Brain	Spleen
2	1	A501	6·6*	R4/2 T103·8 D..	Haemorrhagic gastritis	—	—
4				R8/2 T103·2 D..	Encephalitis.....	+/5·2*	—
10				N.R. D/4.....	Gastritis.....	+/2·7	+
Balance				N.R.....		—	—
12	2	OD	5·0	N.R. D/8.....	Enteritis.....	—	—
13				N.R. D/11.....	Enteritis.....	—	—
18				R8/4 T105·6 D..	Gastritis.....	—	—
Balance				N.R.....		—	—
24	3	L	8·0	R11/4 T105.....		—	—
25				R11/5 T105·4....		—	—
28				R17/2 T104.....		—	—
30				R14/4 T104·2....		—	—
Balance				N.R.....			
31–40	4	Vryheid	6·5	No reactions			
41–50	5	V.H.	7·2	No reactions			
51–60	6	114	7·4	No reactions			
61	7	Karen	7·4	R10/4 T105·6 D..	Encephalitis.....	+/6·4	—
62				R5/8 T105·4 D..	Encephalitis.....	+/4·8	—
63				R8/4 T104·2 D..	Encephalitis.....	+/5·6	—
64				R6/3 T103·2 D..	Encephalitis.....	+/5·8	—
65				R7/6 T106 D..	Encephalitis.....	+/4·0	—
66				R9/6 T106 D..	Encephalitis.....	+/2·6	
67				R8/8 T105·8			
68				R6/4 T103·6 D..	Encephalitis.....	+/5·4	—
69				R7/5 T104 D..	Encephalitis.....	+/4·7	—
70				R7/5 T104 D..	Encephalitis.....	+/4·4	—
71–80	Normal mouse brain			No reactions			

Note.—* Titres expressed as the negative logarithm of the 50 per cent dilution end point.

† R4/2 T103·8 D: Thermal reaction commenced on the fourth day after injection, *two* days later the animal *died*, highest temperature recorded was 103·8°F.

R11/4 T105: Thermal reaction commenced on the eleventh day after injection, persisted for four days, highest temperature recorded was 105°F.

N.R. D/4: No thermal reaction recorded, animal died four days after injection.

at the time, so antigen of known titre was available. Each of a group of 10 guinea-pigs was given 1 ml of infective emulsion of one strain and the injection was repeated on the seventh day. By the fourteenth day, when the third injection was to be given, all ten that had received Karen strain had died. Each guinea-pig that died was examined carefully and it was established without doubt that death was due to a specific aseptic viral encephalitis due to Karen virus. In view of this finding (quite unexpected because, in the light of previous experience, it was anticipated that only the odd animal in either group would die) eight groups of ten guinea-pigs each were selected to receive each of the seven vaccine virus strains, and one group to receive a similar concentration of normal mouse brain emulsion. At the time of injection the virus titre of each inoculum was determined in mice.

The results of this series of injections are summarised in Table 1.

Comment

It will be noted that all ten of the guinea-pigs that received Karen virus showed febrile reactions and nine died after showing symptoms of encephalitis. Virus was isolated from the brain in each instance and except in the case of one guinea-pig (66 titre 2·6) was present in comparatively high titre. In no single instance was virus demonstrable in the spleen.

Of the remaining 60 guinea-pigs which received the other virus strains only one showed symptoms of a fatal encephalitis. This guinea-pig had received virus A501 and the virus shown to be present in the brain in high titre was identified as such by serum virus neutralisation.

As was to be anticipated when working with large numbers of guinea-pigs, which inevitably were somewhat overcrowded, there was some non-specific mortality. Altogether five died showing lesions of gastritis or enteritis. No virus was detected in either brain or spleen except in the case of one that had received strain A501. This guinea-pig, which had shown no signs of ill health, was found dead on the morning of the fourth day after injection. The homologous virus was present in the brain and spleen, in the former in low titre; unfortunately the titre in the spleen was not determined. At this stage no explanation can be offered for this finding.

One further point of interest is apparent. Of the guinea-pigs that received virus Strain L (Group 3), no less than four showed fairly marked febrile reactions after a long incubation period of eleven days or longer. The only symptom was slight inappetence and all recovered.

The remainder of the guinea-pigs showed no reactions and were bled on the thirtieth day for collection of serum.

II. *The effect of the route of infection of guinea-pigs with Karen strain vaccine virus*

Following the observation that the Karen strain of vaccine virus on intraperitoneal injection into guinea-pigs may cause a fatal viral encephalitis in practically 100 per cent of cases, the effect of infection by the intramuscular, subcutaneous, oral, intranasal, rectal and supraconjunctival routes was investigated. The dose in each instance was 1 ml except that 0·2 ml was instilled into the eye.

The results are shown in Table 2.

TABLE 2.—*The effect of the infection of guinea-pigs by various routes with Karen strain vaccine virus*

Guinea-pig number	Titre of Inoculum	Route of Inoculation	Reaction	Virus isolation (Brain)
601	6·3	Intraperitoneal	R7/8 T104·4 D*	+
602			R5/2 T106 D	+
603			R8/7 T105·4 D	+
604			R8/7 T105 D	+
605			R7/8 T103·6 D	+
606	6·3	Intramuscular	R7/7 T103·6 D	+
607			R6/6 T104·4 D	+
608			R9/5 T104 D	+
609			R6/5 T103 D	+
610			R7/5 T105 D	+
611	6·3	Subcutaneous	R7/7 T104·6 D	+
612			R6/3 T106 D	+
613			R8/7 T106 D	+
614			R10/5 T106·4 D	+
615			R7/7 T106·4 D	+
616	6·3	Oral	N.R.	
617			N.R.	
618			N.R.	
619			N.R.	
620			N.R.	
621	6·3	Intranasal	R4/7 T106·4 D	+
622			R3/5 T106·4 D	+
623			R4/3 T106 D	+
624			R3/6 T107·2 D	+
625			R4/5 T107 D	+
626	6·3	Conjunctival	N.R.	
627			N.R.	
628			N.R.	
629			N.R.	
630			N.R.	
761	5·7	Rectal	R9/3 T104·6 D	+
762			R7/5 T104·4 D	+
763			R13/2 T103 D	+
764			R10/3 T104 D	+
765			R6/2 T103·2 D	+
766			R9/3 T103·6 D	+
767			R8/3 T103·4 D	+
768			R6/4 T103·6 D	+
769			R7/3 T103·2 D	+
770			R9/2 T103 D	+

* For Note see Table 1.

Comment

All the guinea-pigs except those infected by the oral and conjunctival routes died after showing symptoms of encephalitis and virus was isolated from the brain in each instance.

Except for the group infected intranasally, the incubation period varied from about seven to ten days and the course varied from about five to eight days. In the case of the group infected rectally, there was a tendency for the incubation period to be lengthened by one or more days, but once symptoms were shown the course of the disease was considerably more rapid. In the intranasal group the incubation period was significantly shorter and the febrile reaction far more pronounced.

In view of the regularity with which infection was set up with this strain of virus by parenteral injection, it was somewhat surprising that intraocular instillation failed. On the other hand the failure of oral administration was to be expected since the sensitivity of this virus to an acid pH is well known (Alexander, 1935).

III. *The infection of guinea-pigs by the intranasal route with the seven vaccine strains of virus and Strain* 7/60

The results of the previous experiment indicated that the intranasal route other than direct intracerebral injection was probably the route of infection of choice, for the infection of guinea-pigs with mouse adapted neurotropic strains of virus. It was decided, therefore, to ascertain the result of the intranasal infection of guinea-pigs with each of the vaccine strains and the attenuated Group 9 virus. For this purpose seven groups each of five guinea-pigs were used for the vaccine strains and one group of ten guinea-pigs for strain 7/60. The results are summarised in Table 3.

Comment

All the guinea-pigs which received the vaccine strains of virus with one exception, guinea-pig 661 (Strain L), showed marked febrile reactions with temperatures up to 108°F. The febrile reactions in the 7/60 virus group were less pronounced.

The period of incubation varied from four to eight days and although it was particularly short in the case of those that received A501, possibly also OD, and was constant at four days in the case of Karen virus, the numbers are too small to warrant any correlation between incubation period and degree of neurotropism.

Of the guinea-pigs that died, virus was isolated from the brain of each and identified by serum virus neutralisation, as the strain used for infection, except in the case of two guinea-pigs that died after infection with Strain 7/60 virus. Since no virus was isolated these two deaths are regarded as non-specific.

If the percentage mortality is regarded as the index of neurotropism, then A501 and Karen viruses (100 per cent mortality) must be regarded as possessing equal affinity for the central nervous system, followed very closely by VH and 7/60 (80 per cent) and thereafter 114 and Vryheid (40 per cent) and L and OD (20 per cent) in that order.

The febrile reaction in the guinea-pigs, which received A501 and Karen viruses, was monophasic. With all other types there was a marked tendency for the fever to be intermittent, the temperature in the terminal stage falling by crisis to subnormal. A striking feature of the symptoms was that, unlike the ascending paralysis that resulted from parenteral injection of virus, the guinea-pigs were depressed from the onset and showed a peculiar tendency to rest their heads on the floor, with no other postural abnormality before coma supervened.

TABLE 3.—*Intranasal infection of guinea-pigs with eight antigenically different strains of mouse adapted neurotropic virus*

Guinea-pig number	Isolate			Reaction*	Virus reisolation (Brain)
	Type	Strain	Titre		
651	1	A501	7·5	R2/11 T105 D	+
652				R3/8 T105·6 D	+
653				R3/7 T106·2 D	+
654				R2/13 T105·8 D	+
655				R2/13 T104·6 D	+
656	2	OD	6·5	R4/19 T104	
657				R3/21 T105	
658				R2/15 T106·2	
659				R5/5 T105	
660				R4/15 T105·6 D	+
661	3	L	7·3	N.R.	
662				R7/10 T105	
663				R6/10 T106	
664				R6/11 T104·6	
665				R6/15 T106 D	+
666	4	Vryheid	7·4	R6/17 T105·2	
667				R8/11 T104·6 D	+
668				R5/15 T106 D	+
669				R6/16 T105	
670				R6/17 T105	
671	5	V.H.	7·5	R4/9 T105·6 D	+
672				R5/9 T105·2 D	+
673				R5/11 T105·4 D	+
674				R5/9 T105·4 D	+
675				R4/7 T104	
676	6	114	8·0	R5/6 T106	
677				R4/9 T105 D	+
678				R4/8 T104	
679				R4/6 T104·6	
680				R5/10 T105·2 D	+
681	7	Karen	7·4	R4/7 T108 D	+
682				R4/8 T107 D	+
683				R4/8 T106 D	+
684				R4/8 T107·2 D	+
685				R4/7 T106 D	+
771	9	7/60	7·0	R17/6 T103	
772				R6/7 T104·8 D	+
773				R6/4 T103 D	+
774				R6/10 T104 D	+
775				N.R. D/14†	−
776				R3/14 T105	
777				R6/4 T104 D	+
778				N.R. D/3‡	−
779				R5/8 T104·6	
780				R8/7 T103·4 D	+

* See Note, Table 1.

† Died from extensive liver abscessation.

‡ Died from acute gastro-enteritis.

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IV. *The antigenicity of the vaccine virus strains*

The guinea-pigs, which survived the intraperitoneal injection of virus in the first experiment, were bled by cardiac puncture on the thirtieth day, with the exception of the single survivor in the Karen group, which was challenged by intraperitoneal injection of the homologous virus to which it showed no reaction and was bled after a further interval of thirty days. The sera of the guinea-pigs in each group were pooled for determination of the antibody titre against the homologous virus by serum virus neutralisation. The results are summarised in Table 4.

TABLE 4.—*The antibody titre of the sera of guinea-pigs that survived the intraperitoneal injection of vaccine virus strains*

	Antigen and virus titre in test						
	A501/125	OD/40	L/40	Vryh./80	V.H./36	114/150	Karen/40
Antibody titre	106*	172	3125	0	66	56	3125†

* Antibody titre expressed as the reciprocal of serum dilution end point.

† Serum collected after two inoculations with homologous virus (Guinea-pig No. 67).

Comment

The point of importance that is apparent from the results of this experiment is the fact that the virus neutralising antibody titre of the pools of guinea-pig sera varied within exceedingly wide limits. Admittedly, in the case of Karen virus, only one guinea-pig was involved and it received two injections of virus so that the high titre antibody may be due to a booster effect in a particularly responsive individual. On the other hand, equally high titre antibody was present in the pooled sera of ten guinea-pigs that received a single injection of L virus and no antibody could be detected in a pool of ten guinea-pigs that received a single injection of Vryheid virus. It is concluded, therefore, that the antigenicity of different strains of mouse adapted neurotropic virus as determined by the response to intraperitoneal injection of guinea-pigs is extremely variable.

DISCUSSION

It was known that all the strains of horsesickness virus attenuated for the immunisation of horses and mules by serial intracerebral passage in mice, invariably produce a fatal encephalitis in guinea-pigs infected by intracerebral injection. It was known that at least one such strain, on intracerebral injection into horses, produced only a transient encephalitis followed by the development of immunity. It has also been shown that several such strains on extraneural parenteral injection into guinea-pigs produced no more than a variable but small percentage mortality from encephalitis. Since the immunisation of equines in the field in South Africa on a large scale over a period of many years, first with a quadrivalent vaccine and subsequently with polyvalent vaccines, produced no evidence either of neuroparalytic accidents or the ability of virus strains to penetrate the blood brain barrier with fatal results in equines, it was assumed that this innocuity was shared by all attenuated strains of virus. The first indication that this assumption is untenable was the report

of some mortality amongst donkeys, mules and horses, in that order of frequency, following the use of routine polyvalent vaccine on a fully susceptible equine population for the control of the disease after its introduction into the Middle East (Orhan, 1961; Huq, 1961; Kaveh, 1961; Nobel & Neumann, 1961).

The data presented in this preliminary report show that while some strains of attenuated virus on intraperitoneal injection into guinea-pigs may produce no detectable clinical reaction (Strains OD, Vryheid, VH and 114), or only a well marked non-fatal febrile reaction (L) or limited mortality from encephalitis (A501) followed by the appearance of antibody in the serum, at least one strain (Karen) may be relied upon to produce almost 100 per cent mortality from aseptic viral encephalitis. This ability of the Karen strain of attenuated virus to invade the central nervous system, follows infection by other routes, e.g. the subcutaneous, intramuscular, rectal and intranasal routes but not the oral or conjunctival.

From the results of the infection of guinea-pigs by the intranasal route with an attenuated strain, representing each of eight antigenically different groups of virus, it is apparent that this is the most sensitive method of demonstrating relative degrees of neurotropism in this laboratory animal, in that at least one guinea-pig in each group succumbed to encephalitis, whereas there were no specific deaths among guinea-pigs that received five virus strains intraperitoneally (OD, L, Vryheid, VH and 114). Moreover, in the case of Strain A501, when virus was demonstrated in the brain of only two out of ten guinea-pigs after intraperitoneal injection five out of five succumbed to intranasal infection. If the percentage mortality following intranasal infection is regarded as the index of neurotropism then the eight strains investigated may be classified in descending order as Karen and A501, followed closely by VH and 7/60, and thereafter 114 and Vryheid, and L and OD. There remains to be established a correlation between the degree of neurotropism as indicated by intraperitoneal and/or intranasal infection of guinea-pigs and the ability to produce either a fatal encephalitis on direct intracerebral injection into susceptible donkeys, mules and horses or its ability to penetrate the blood-brain barrier on extraneural parenteral injection. In addition, it is essential to establish whether the degree of neurotropism is enhanced for equines as for laboratory animals by prolonged passage or whether it is an inherent property peculiar to each different wild virus strain. Meanwhile it is suggested that use of the guinea-pig may be a valuable method of screening attenuated virus strains or combinations of strains for innocuity, prior to incorporation in any polyvalent vaccine.

The virus neutralising antibody response of guinea-pigs to the various vaccine virus strains is the second point of considerable importance. The results of the single experiment reported, emphasise the great difference in the response to the parenteral injection of guinea-pigs with antigenically different vaccine virus strains. Current investigations show that the results are not fortuitous, but are reproducible quantitatively and that there is a considerable variation in antigenicity not only in strains from different groups, but also in strains within a group. That there may be a clearly defined correlation between antigenicity in guinea-pigs and horses is suggested by the work in progress and is supported by the findings of Howell (1963) in regard to the response of horses to repeated polyvalent immunisation. Without entering into any discussion of the significance of the virus neutralising antibody titre of the serum as an index of artificially induced resistance to natural infection in the horse, it is suggested that use of the guinea-pig may be a method of great value as the basis of a screening test for the selection of strains of virus of high antigenic potency for routine immunisation.

A point of minor importance but some interest emerges from the successful infection of guinea-pigs by the rectal and intranasal routes. All evidence supports the belief that in the case of equines transmission of infection never takes place by direct or indirect contact. However, intranasal infection appears to be a possible explanation for the incidence of fatal infections in dogs fed the uncooked flesh of horses that had died of horsesickness, and serves as a warning that care should be taken to exclude accidental infections in small laboratory animals, for instance by means of a contaminated thermometer.

In conclusion it is desired to point out that this preliminary report is presented in the hope that it may be of value to the comparatively limited number of workers in this particular field which is so vast and complicated by the plurality of virus strains encountered in nature.

Summary

Attention is directed to the lack of a readily available small laboratory animal to replace the horse in evaluating the innocuity and antigenicity of strains of horsesickness virus attenuated for equines by serial intracerebral passage in mice.

In guinea-pigs the result of the intraperitoneal injection of vaccine virus strains may range from the absence of any clinical reaction to the production of 100 per cent mortality from specific aseptic viral encephalitis.

Guinea-pigs are susceptible to infection and the production of encephalitis by the intraperitoneal, subcutaneous, intramuscular and rectal routes of infection, but not *per os* or by supraconjunctival instillation.

The intranasal route appears to be the most constant one for the invasion of the central nervous system by virus.

Recovery from infection is followed by the appearance of serum virus neutralising antibodies in the serum, the titre varying with the antigenic potency of the particular attenuated virus strain.

The value of the guinea-pig in future research into horsesickness is discussed.

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