EFFECT OF CERTAIN MURINE PATHOGENS ON PHAGOCYTIC ACTIVITY

A. W. GLEDHILL, D. L. J. BILBEY AND JANET S. F. NIVEN

From the National Institute for Medical Research, Mill Hill, London, N.W.7 and the Department of Anatomy, King's College, University of London, London, W.C.2

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THE activity of the reticulo-endothelial system in the removal of particles, including microorganisms, from the circulating blood has been well established. Furthermore it has been shown that over 90 per cent of the cells of this system are situated in the liver as the littoral macrophage or Küpffer cells (Halpern, Biozzi, Nicol and Bilbey, 1957). Mims (1959*a*, *b*) reported that a high proportion of virus particles inoculated intravenously are taken up by the Küpffer cells of the liver. For example, he observed that ectromelia virus, which produces extensive destruction of liver parenchyma cells, first infects Küpffer cells and is not found in the parenchyma cells until a cycle of virus growth within the Küpffer cells has been completed. Other workers have also indicated similar host-virus activity. An early effect of infection of mice with lymphocytic chorio-meningitis virus (LCM) is destruction of lymphocytes and focal destruction of some cells of the reticulo-endothelial system in liver and spleen, followed by damage to liver parenchyma and red pulp of the spleen (Niven, unpublished). Similarly, virulent mouse hepatitis virus appears to enter and multiply in Küpffer cells before it infects and destroys liver parenchyma cells (Bang and Warwick, 1959 ; Boss and Jones, 1963). These observations suggest that such viruses infect and multiply within phagocytic cells before involvement and destruction of parenchymal cells can occur. Thus it is of practical importance to know the fate of phagocytosed virus particles, their effect on host macrophage cells and their subsequent effects on neighbouring tissues.

Infection of mice with the lactose dehydrogenating virus (Riley, Lilly, Huerto and Bardell, 1960) has been found to depress their capacity to remove carbon particles from the blood and the depression extends from one until about 3 days after infection (Notkins and Scheele, 1964 ; Mahy, 1964). Since infection of phagocytes might impair their phagocytic activity we investigated the capacity of mice infected with the above mentioned "hepatitis" viruses to phagocytose carbon particles in the blood and compared the phagocytic capacity (expressed as index K) with that of uninfected mice. As expected, infection with these viruses was found to depress phagocytosis. Similar determinations of phagocytic index were made for mice infected with agents that do not cause hepatitis. One such agent (PR8 strain of influenza A virus) had little effect upon phagocytosis, while others, the Friend and Moloney leukaemia viruses, increased the phagocytic index to a level well above the normal. Furthermore, since the murine parasite, *Eperythrozoon coccoides* (Schilling, 1928) raises the virulence of mouse hepatitis viruses we also investigated the effect of this agent alone and in combination.

METHODS

Mice.—Mice of 12–14 g. of the VS strain bred at the National Institute for Medical Research since 1949 were used unless otherwise stated. In all experiments, some mice were infected with the agents listed below and others were kept as controls. At predetermined times after infection, the phagocytic indices of infected animals were determined and compared with those of the controls.

Determination of phagocytic index K.—The general procedure for determination of phagocytic index was an estimation of the rate of removal from the blood-stream of carbon particles (diameter 250 Å) injected intravenously in a dose of 16 mg. per 100 g. body-weight, broadly in accordance with the technique described by Nicol, Bilbey and Ware (1958). Blood samples were drawn from the intra-orbital sinus at known times (in minutes) after the carbon injection and lysed in sodium hydroxide solution. A graph of the logarithm of absorptiometer readings made with the samples against the times after injection yields a straight line whose slope represents the phagocytic index or K-value.

Agents and dosage.—Eperythrozoon coccoides, obtained originally from a strain of mice bred at Mill Hill, is maintained by weekly passage in VS mice by intraperitoneal (i.p.) passage of heparinized blood diluted 10^{-6} and checked by examination of Giemsa stained blood smears. Experimental mice were similarly infected with fresh, weekly passage blood.

Titrated liver spleen suspensions of virulent mouse hepatitis virus (MHV3) and avirulent mouse hepatitis virus (MHV1), stored in ampoules at −70°, were diluted as required in serum broth saline (SS broth : 45 per cent normal saline, 45 per cent broth, 10 per cent horse serum) and inoculated i.p. into mice in 0·2 ml. amounts. MHV3 was diluted to give 30 LD_{50} and MHV1 1000 LD_{50} per 0·2 ml. (as determined by titration in *E. coccoides* pre-infected mice).

Lymphocytic choriomeningitis virus (LCM), strain UBC, supplied by Dr. J. Seamer as a brain suspension containing $10^{7.4}LD_{50}$ per g. brain, was inoculated into groups of mice subcutaneously (s.c.) and intracerebrally (i.c.) in doses of 0·03 ml. containing 3000 LD_{50}. Similar mice were inoculated with normal mouse brain suspension of the same concentration to control the effect of brain tissue on phagocytic activity. Dilutions of brain suspension for intracerebral inoculation were made in normal saline containing 1 per cent horse serum.

Ectromelia virus strain MH (Gledhill, 1959) maintained at −70° was diluted in SS broth and inoculated i.p. into mice in doses of 0·2 ml. liver spleen suspension containing 100 LD_{50} of virus. With this dose many mice appear ill 6 days after inoculation and die the following day but some survive considerably longer.

The murine leukaemia viruses (Friend and Moloney) were supplied as lyophilised suspension by Dr. Charlotte Friend and Dr. J. B. Moloney. After a few passes in Albany strain mice at the New York State Laboratories, Albany, N.Y., and a number of passes in VS mice at Mill Hill, batches were stored at −70° as 10 per cent suspensions of spleen in the case of the Friend virus and mixed spleen, lymph node and thymus in the case of the Moloney virus. For experiments with the Friend virus, i.p. inoculations (0·2 ml.) of 1 per cent suspension were made into VS mice of 12–14 g., while for experiments with Moloney virus, i.p. inoculations (0·03 ml.) of 10 per cent suspension were made into day old VS mice. In the latter case, measurements of phagocytic index were not made until the mice reached 21 days of age, when their weight varied from 7–11 g.

Influenza A virus (PR8) was obtained as allantoic fluid from Dr. H. G. Pereira. It was inoculated intranasally into mice (anaesthetised with ether) in doses of 0·03 ml. in dilutions (made in SS broth) of 10^{-4}, 10^{-3} and 10^{-2}. The two lower dilutions produced fatal pneumonia, while the higher dilution was not lethal.

RESULTS

Viruses that produce hepatitis

Phagocytic indices were determined for groups of 5 mice at approximately the middle and the end of the incubation period following infection with ectromelia virus, MHV3 and MHV1. For ectromelia virus the indices were determined at 3 and 6 days after infection, for MHV3 at 2 and 4 days and for MHV1 at 3 and 7 days. The results are shown in Table I. It will be seen that the phagocytic index for mice infected with ectromelia virus is much below that of normal mice at the time

TABLE I.—*Phagocytic Index of Mice Infected with Hepatitis Viruses*

	K-Values								
	Controls		Mid-incubation period			Onset of illness		
Virus	Mean	Range	Mean	Range	Result	Mean	Range	Result
Ectromelia	19	15–25	17	14–19	ns*	5	4–5	§§§§ ($P<0\cdot004$)
MHV3	24	18–29	8	4–12	§§§ ($P<0\cdot004$)	10	6–18	§§§ ($P<0\cdot008$)
MHV1	28	21–32	25	18–28	ns	24	17–32	ns

* ns = K-value not altered (within 20 per cent of normal]
§§§ = K-value 41–60 per cent ,,
§§§§ = ,, 60 per cent ,,

when illness becomes apparent but is not significantly reduced at the mid-incubation period, while for mice infected with MHV3 there is significant reduction even during the incubation period. In contrast, the phagocytic index of mice infected with MHV1, which causes only mild liver damage, is not significantly reduced. Lymphocytic choriomeningitis virus produces a lethal disease when inoculated by the intracerebral route (i.c.) and a non-lethal disease when inoculated subcutaneously (s.c.). Mice were inoculated by these routes with a mouse brain suspension containing this virus, as indicated under " Methods ", and phagocytic indices were determined in groups of 2–4 mice 3 and 6 days after inoculation. As already noted, control mice received by the same routes suspensions of similar concentration of normal brain, a necessary condition since normal brain suspension produces a transient rise of phagocytic index. The results presented in Table II show that

TABLE II.—*Phagocytic Index of Mice Infected with LCM Virus s.c. and i.c.*

		K-Values									
		Normal brain					LCM brain					
Route	Time after inoculation (days)	1	2	3	4	Mean	1	2	3	4	Mean	Result
s.c.	3	32,	34,	36,	41	36	32,	34,			33	ns*
s.c.	6	21,	29,	32		27	17,	18			17	§§ ($P = 0\cdot1$)
i.c.	3	35,	40,	47,	53	45	26,	33,	48		35	§ ($P = 0\cdot14$)
i.c.	6	24,	25,	25		25	14,	14,	15,	23	16	§§ ($P = 0\cdot03$)

* = K-value not altered (within 20 per cent of normal)
§ = K-value 20–30 per cent depressed
§§ = ,, 31–40 per cent ,,

6 days after infection with LCM virus a moderate depression of phagocytic index occurs with mice infected by either route ; the table also shows that 3 days after intracerebral infection there is some depression, but the result is not statistically significant. Phagocytic indices were somewhat elevated 3 days after inoculation of normal brain suspension by either route but returned to normal values by the sixth day.

Avirulent mouse hepatitis (MHV1) and Eperythrozoon coccoides

The fact that the mild hepatitis produced by MHV1 was associated with much less effect upon phagocytic activity than lethal MHV3 infection prompted us to determine the effect upon phagocytosis of the lethal hepatitis that results from infection with MHV1 and *E. coccoides* (Niven, Gledhill, Dick and Andrewes, 1952). A group of 12 mice were inoculated i.p. with a 10^{-6} dilution of blood containing *E. coccoides*. Two days afterwards, 7 of these mice were inoculated i.p. with MHV1, while the remainder were set aside as inoculated controls. At the same time, 5 normal mice were infected with MHV1 to serve as additional controls. The phagocytic indices of the mice of these 3 groups and a group of 5 uninoculated mice were determined 4 days after the time of MHV1 inoculation, this last group providing the essential base line for assessment. The results presented in Table III

TABLE III.—*Phagocytic Index of Mice Infected with* E. coccoides, MHV1 *and Both Agents*

Agent	Phagocytic indices Individual mice	Mean	Result	Significance (probability)
Nil	18, 23, 23, 26, 29	24	—	—
MHV1	13, 15, 19, 21, 24	18	25 per cent depressed	0·05
E. coccoides	57, 60, 62, 68, 70	63	160 per cent elevated	0·004
MHV1* + *E. coccoides*	6, 11, 11, 12	10	58 per cent depressed	0·008
	35, 37, 51	41	70 per cent elevated	0·018

* *E. coccoides* (E.c.) inoculated i.p. as 10^{-6} dilution of infected blood followed in 48 hr. by MHV1 i.p. as 1 per cent liver suspension.

show that 6 days after inoculation the phagocytic indices were greatly increased in mice of the group infected with *E. coccoides* only. The K-values for the mice infected with MHV1 alone showed a small but significant depression. The indices for the mice inoculated with *E. coccoides* and with the virus 2 days afterwards fell into 2 groups, 4 with severe depression and 3 with moderate elevation. Those that were depressed gave values of the same order as on the fourth day of infection with virulent MHV3 (Table I) and those that were elevated were significantly less elevated than the indices of mice infected with *E. coccoides* alone.

The fact that the phagocytic index of some mice of the group inoculated with both agents was depressed while that of others was elevated calls for comment. Infection with *E. coccoides* only begins to raise the pathogenicity of MHV1 after 3–4 days when parasitaemia is readily demonstrable (Gledhill, 1956) and, at this stage, K-values are found to be elevated. From this point, the MHV1 starts to behave as a virulent agent and the elevated phagocytic index starts to fall. As the disease progresses, the phagocytic index falls further and reaches a low level at the time when the mice are manifestly ill. However, both the progress of the *E. coccoides* infection and that of the virus infection are not constant for each mouse. Consequently, at the time when illness within a group is obvious, disease has advanced further in some individuals than in others and the degree of depression of phagocytic index below that associated with *E. coccoides* infection may be expected to vary with the stage of pathological change in each mouse. In another experiment 8 mice, infected with *E. coccoides* and with MHV1 the following day, were tested at a later stage of infection than in the previous experiment, namely, 5

days after virus inoculation. As controls, 5 mice infected with *E. coccoides* alone, 5 mice infected with MHV1 alone and 3 normal mice were tested. The phagocytic indices of these animals are shown in Table IV.

TABLE IV.—*Phagocytic Indices of Control Mice*

Group of mice	K-values	Mean
Normal (3)	40, 36, 34	37
MHV1 (5)	53, 43, 41, 36, 31	41
E.c. (5)	140, 138, 126, 109, 87	120
E.c.+MHV1	16, 13, 12, 11, 10, 10, 9	12 } 20
	82	(82) }

It will be noted the indices of 7/8 mice of the group inoculated with both agents were depressed far below the normal and only one was elevated, whereas 4 were depressed and 3 elevated in the first experiment. This difference is due to the fact that the disease was more advanced at the time of test in the second experiment.

E. coccoides

The great elevation of the phagocytic index of mice 6 days after infection with *E. coccoides* was confirmed and extended. Half of a group of 36 mice were infected with *E. coccoides* as before and the other half left as controls. Determinations of phagocytic index at various times after infection were made in groups of 3 infected and 3 control mice. It will be seen from Table V that the K-values were eleva-

TABLE V.—*Effect of* E. coccoides *Infection on Phagocytic Index*

Mean K-Value	Days after *E. coccoides* infection					
	3	4	5	6	7	11
Infected Mice	18	35	44	55	60	96
Normal ,,	15	18	18	20	16	18

ted by the 4th day after infection and continued to rise until the termination of the experiment on the 11th day. The parasite is known to be present in the blood in maximum amount around the 5th day and has considerably declined by the 11th day. Thus, it would appear that the K-value continues to rise even after the concentration of parasites in the blood has begun to fall.

Mouse leukaemia agents

Prior infection of mice with the Friend and Moloney leukaemia agents raises the pathogenicity of MHV1 in a way similar to infection with *E. coccoides* (Gledhill, 1961). It seemed possible therefore that infection with these leukaemia agents might also resemble *E. coccoides* infection in its influence on the phagocytic index.

A group of mice were inoculated i.p. with a 1 per cent spleen suspension containing the Friend agent and an equal number of similar mice were kept as controls. The phagocytic index was determined for groups of 3 infected and 3 normal mice at intervals and the results obtained are indicated in fig. 1. It will be seen that 7 days after infection the mean index for infected mice was elevated above the control range. It continued to rise to a value in excess of 100 on the 15th day but returned to a normal value by the 24th day. Further experiments in which

dilutions of the Friend agent were inoculated into mice confirm that the phagocytic index is greatly elevated on the 12th day after infection.

In the case of the Moloney agent, a 1 per cent mixed tissue suspension was inoculated into half the mice of each of 4 litters of day-old mice, the uninoculated half litters being left as contacts. Determinations of phagocytic index were made on infected and contact mice 3 weeks after infection, the period at which the Moloney agent is able to raise the pathogenicity of MHV1 (Gledhill, 1961). Owing to the smallness of the mice at this age and to secure maximum sensitivity, a larger dose of carbon than usual (32 mg./100 g. body weight) was employed in these tests and for this reason the K-value for normal mice was expected to be about 14 instead of 24. The result obtained appears in Table VI and shows that

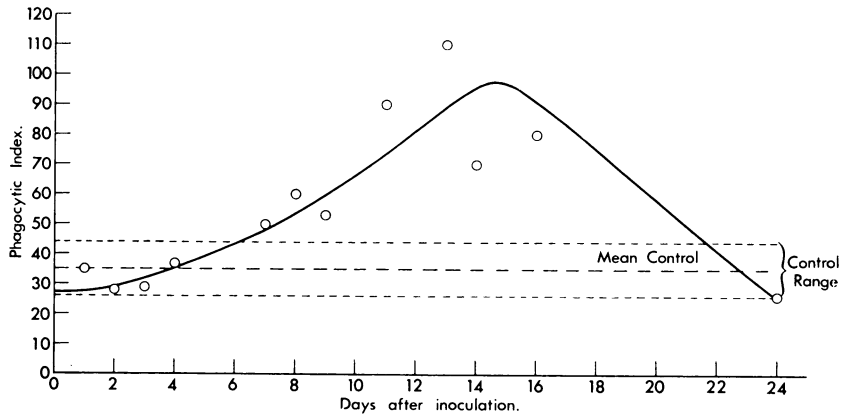

FIG. 1.—Relationship between phagocytic index and time of infection with Friend virus.

TABLE VI.—*Elevation of Phagocytic Index in Moloney Infected Mice and Controls*

Class of mice	Phagocytic indices Mouse No. 1	2	3	4	5	Mean		Significance
Controls .	13	13	14	15	—	14	No sig. diff.	Significant elevation $P=0{\cdot}0002$
Contacts .	13	14	16	16	—	15		
Moloney .	18	20	22	24	25	22		

the phagocytic index of the Moloney infected mice is regularly although only moderately raised in comparison with the controls. Furthermore phagocytic indices of the contacts are indistinguishable from those of the controls. The probability that the contacts and Moloney infected mice belong to the same population is 0·0011 and so, presumably, spread of infection from infected mice to contacts did not occur. In another experiment the virus was inoculated i.p. into litters of day-old mice at dilutions from 10^{-1} to 10^{-9}. Twenty-one days afterwards, the phagocytic index was determined for 3 mice of each litter and 3 uninoculated mice of the control litter. The results presented in fig. 2 show that the clearance of carbon from the blood of mice infected with dilutions of virus from 10^{-1} to 10^{-6} was accelerated and gave lines totally outside those from normal mice. The lines for dilutions of 10^{-8} and 10^{-9} fell amongst the control lines, while 10^{-7} fell

outside but close to the control lines. The individual K-values are set out in Table VII. The mean for mice of the control group and two highest dilutions (10^{-8} and 10^{-9}) is 23·3 with standard deviation 2·5. If mice of these groups are assumed to be uninfected and if mice with K-values more than two standard deviations above 23·3, i.e. greater than 28·3, are regarded as infected, an infection rate related to dilution becomes clearly apparent. Using the method of Reed and Muench (1938), the result indicates an ID_{50} of $10^{6.88}$ per g. leukaemic tissue. The tested mice and other infected mice were regularly examined, and killed and autopsied

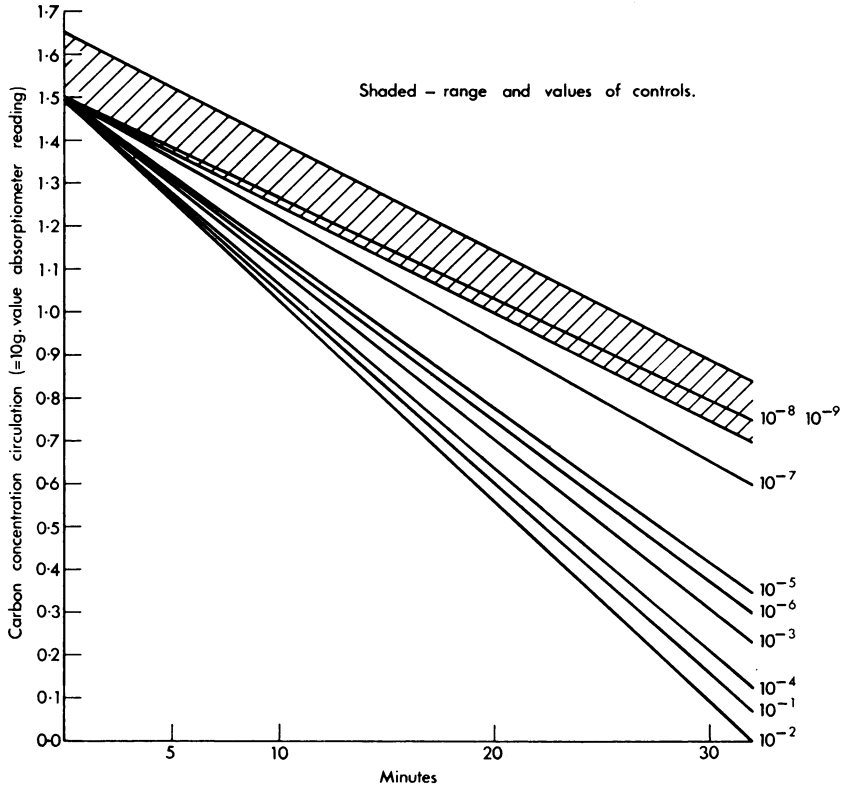

FIG. 2.—Rate of removal of carbon from the blood of mice infected with dilutions of Moloney virus.

when signs of leukaemia became evident. The residue were killed after 176 days. Unfortunately, at this time leukaemia had developed in only 5 mice, one each at dilutions 10^{-5} and 10^{-3} and 3 at dilution 10^{-1}. From limited observation, only a minority of mice of the VS strain, unlike mice of certain other strains, develop leukaemia after infection with the Moloney agent. The probability that leukaemia would not occur in any mice with normal K-values, i.e. the control and high dilution groups (0/23), and yet occur in 5 mice of the low dilution groups with elevated K-values (5/27) is a little less than 0·04. Thus, the observed incidence of leukaemia does not conflict with the titration based on elevation of phagocytic index, provided it is conceded that only a small proportion of infected mice developed leukaemia.

TABLE VII.—*Effect of Dilutions of Moloney Agent on Phagocytic Index*

Log dilution of Moloney agent*	K-Values Mouse 1	2	3	4	Proportion mice infected
∞ (Control)	21	22	26	28	0/4
9	19	23	25	—	0/3
8	23	23	23	—	0/3
7	25	27	*32*†	—	1/3
6	*36*	*38*	*40*	—	3/3
5	27	*35*	*45*	—	2/3
4	*43*	*43*	*44*	—	3/3
3	*38*	*40*	*42*	—	3/3
2	*42*	*43*	*56*	—	3/3
1	*40*	*45*	*48*	—	3/3

* Inoculum 0·03 ml i.p. into day old VS mice and K-values determined 3 weeks later.
† Mice with K-value >28 regarded as infected (indicated by italics). ID_{50} per g. tissue $10^{6.88}$.

Effect of influenza A virus (PR8) on phagocytic index

Since the hepatitis viruses studied depressed phagocytosis and the agents that raise the pathogenicity of MHV1 elevated phagocytosis, the effect of a virus unassociated with hepatitis and unable to raise the pathogenicity of MHV1 was studied. A strain of influenza virus was selected for this purpose since it multiplies in the respiratory system after intranasal instillation without detectable viraemia or widespread dissemination in the reticulo-endothelial cells of the body. The phagocytic index of mice infected by intranasal instillation of allantoic fluid, as stated under " Methods ", was determined on the 1st, 2nd and 3rd day after infection at dilutions which gave minimal lung lesions or fatal pneumonia. In the latter case the mice were obviously ill when tested and were dead on the day following the last test. In both the mild and fatal disease the phagocytic indices were slightly but consistently raised above those of the controls (Table VIII).

TABLE VIII.—*Phagocytic Index of Mice Inoculated with Influenza A Virus*

Dilution of PR8	Mice in group	Mean K-values Days after infection *1*	*2*	*3*
10^{-4}	8	33	46	34
10^{-3}	9	31	32	34
10^{-2}	9	32	33	36
Controls 1	6	28	—	—
,, 2	6	25	—	—

The interpretation of the difference between the phagocytic indices of the infected and control mice, a difference of a lower order than most of the differences previously associated with infections, is not clear.

DISCUSSION

Jenkin and Rowley (1961) have shown that removal from the blood of inert particles such as those of carbon and quartz depends upon serum opsonins. Consequently, it could be argued that in the above experiments we measured the effects of virus infections upon serum opsonins rather than a direct effect upon phagocytic cells. This seems unlikely to be the major cause of the changes in the

rate of carbon phagocytosis observed, although it is a factor that must not be overlooked. Amongst the viruses studied, only those that produce hepatitis were found to depress phagocytosis of carbon particles but experience with many more viruses and in animal species other than mice would be necessary to establish such a correlation. Indeed, it may be that some viruses multiply in reticulo-endothelial cells without leading to extensive necrosis of liver parenchyma cells and yet depress the phagocytic index. Lymphocytic choriomeningitis virus may be an example in which infection of phagocytic cells precedes involvement of cells in tissues additional to the liver. A degree of liver damage always occurs following inoculation with LCM virus and this is increased when the virus is combined with *E. coccoides*. However, the lethal effect produced by inoculating subcutaneously a combination of *E. coccoides* and LCM virus is not necessarily due to hepatitis alone ; it is more likely to be the result of an increased production of virus peripherally in mice infected with *E. coccoides* causing damage to blood vessels generally and especially those in the CNS (Niven, unpublished). In the case, however, of the increased pathogenicity of MHV1 for mice infected with *E. coccoides*, the lethal result is due to a massive destruction of the liver parenchyma. The view has been advanced that *E. coccoides* may not directly raise the susceptibility of liver parenchyma cells to destruction by MHV1 but rather that it raises the susceptibility of Küpffer cells and thereby permits the liver parenchyma cells to be readily invaded by the virus (Gledhill, 1962). This view of the pathogenesis is supported by the present observation that MHV1 produces only a small depression of the phagocytic index when it acts as a benign hepatitis agent and yet depresses it markedly when it acts as a lethal hepatitis agent in the presence of *E. coccoides*. Moreoever, the observation that *E. coccoides* and the murine leukaemia viruses which raise the pathogenicity of MHV1 are agents that also raise the phagocytic index of mice far above the normal also suggests that the pathogenicity of MHV1 is raised when the phagocytic properties of the Küpffer cells are increased. It may be added that Roberts (1963) has suggested that virulent and avirulent strains of ectromelia virus are equally destructive to liver parenchyma cells and that the difference between them resides in the increased capacity of virulent strains to infect and multiply in Küpffer cells.

The observation that the agents which raise the pathogenicity of MHV1 also stimulate phagocytosis might suggest increased phagocytosis as the explanation of the increased pathogenicity. However, caution in acceptance of this explanation is necessary since we have found that weanling mice inoculated with 5 daily doses of 0·5 mg. stilboestrol, mice in which the K-values are markedly stimulated (Nicol and Bilbey, 1957), possess no increased susceptibility to the pathogenic action of MHV1.

The increase of phagocytic index resultant upon infection with the murine leukaemia viruses tested could have practical implications. If such increases are produced by other murine leukaemia viruses and not by the viruses that are apt to contaminate murine tissue suspensions, then measurements of phagocytic index could be used as an indicator of infection of mice long before leukaemia would be expected to develop. A similar method was advocated for the early recognition and titration of leukaemia viruses by the capacity of such agents to increase the pathogenicity of MHV1 (Gledhill, 1961). The method herein suggested could prove both more sensitive and more practicable than that which depends upon raising the pathogenicity of MHV1. It also offers the advantage that the test does

not require the mice to be killed ; indeed tested mice can be kept long enough to determine whether leukaemia develops.

SUMMARY

Mice infected with virulent hepatitis (MHV3), ectromelia and lymphocytic choriomeningitis viruses, agents known to be taken up by the Küpffer cells before involvement of the liver parenchyma, manifest a reduced capacity to remove injected carbon particles from the blood, the rate of removal being expressed as the phagocytic index.

Reduction of phagocytic index of mice infected with mouse hepatitis virus of low virulence (MHV1) was slight.

Since MHV1 acts as a virulent hepatitis virus when combined with the murine parasite *Eperythrozoon coccoides* the effect of the combination upon the phagocytic index was studied. Infection with *E. coccoides* alone raises the phagocytic index far above normal values. Infection with both the virus and parasite leads to depression of the phagocytic index of some mice, while in others it is raised but to a lesser extent than in mice infected with *E. coccoides* alone. Depression in some mice with elevation in others is considered to reflect the variable stage of the complex disease reached by individual mice at the time the phagocytic index is determined.

The phagocytic index of mice was raised by infection with the Friend and Moloney leukaemia viruses, agents known to enhance the pathogenicity of MHV1.

Application of the method especially in relation to virus diseases of long incubation period is discussed.

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