Br. J. exp. Path. (1969) **50**, 475.

RELATIONSHIP OF PHAGOCYTIC ACTIVITY TO PATHOGENICITY OF MOUSE HEPATITIS VIRUS AS AFFECTED BY TRIOLEIN AND CORTISONE*

G. C. LAVELLE† AND T. J. STARR‡

From the Lobund Laboratory, Department of Microbiology, University of Notre Dame, Notre Dame, Indiana 46556

Received for publication April 10, 1969

SUMMARY.—The pathogenicity of mouse hepatitis virus (MHV-1) was studied following treatment with triolein and cortisone which, respectively, stimulated and suppressed phagocytic activity of the reticuloendothelial system (RES) as measured by clearance of colloidal carbon. When inoculated i.p., triolein moderately enhanced RES activity of germfree mice, while exerting no significant effect in conventional mice. In both groups of mice, however, protection was found against an i.p. challenge of virus. Cortisone greatly suppressed RES activity and significantly increased susceptibility of germfree and conventional mice to MHV-1. Triolein also was found to protect mice against the combined challenge of cortisone plus virus. However, triolein, whether injected i.p. or i.v., failed to protect against an i.v. challenge of virus. These data support two conclusions: (1) triolein exerted its protective effect at some site other than the macrophages of the liver; (2) protection against MHV-1 infection following triolein treatment was not related to the carbon clearing activity of the RES.

MACROPHAGES of the liver (Kupffer cells) are of particular interest in the study of murine hepatitis (MHV) virus infections since it has been demonstrated *in vitro* and *in vivo* that infection of Kupffer cells by these viruses precedes involvement of hepatic cells (Bang and Warwick, 1960; Ruebner and Miyai, 1962). Reports from several sources (reviewed by Mims, 1964) have suggested that, for those viruses which are capable of infecting Kupffer cells, rapid phagocytosis may hasten the pathogenic process.

In previous studies (Lavelle and Starr, 1968*a*), germfree mice were shown to be more susceptible than conventional counterparts to i.v. challenge of MHV-1. However, differences in mortality were not believed attributable to differences in susceptibility of Kupffer cells, as measured by virus titres. The present study concerns the relationship between phagocytic activity of Kupffer cells and pathogenesis of MHV-1. Two agents with known effects on clearance of carbon by the RES have been used. The first of these, glyceryl trioleate (triolein) is a RES stimulant (Stuart, Biozzi, Stiffel, Halpern and Mouton, 1960) which was shown by Vella and Starr (1965) to increase the resistance of germfree mice to a combined cortisone-MHV-1 challenge. The second, cortisone, is a RES suppressant (Biozzi,

This paper is dedicated to the memory of Dr. A. W. Gledhill.

* Supported by the Office of Naval Research, Washington, D.C., Grant No. 1623(14).

† Present address: Department of Pathobiology, School of Hygiene and Public Health, Johns Hopkins University, Baltimore, Maryland 21205.

‡ Present address: Department of Biological Sciences, University of Illinois at Chicago Circle, Chicago, Illinois 60680.

Benacerraf and Halpern, 1955) and increases the susceptibility of mice to MHV-1 (Starr and Pollard, 1958). Since microbes and microbial products are also known to influence RES activity, we were interested in comparing responses of germfree mice with their conventional counterparts.

MATERIALS AND METHODS

Animals.—Swiss-Webstergerm free mice and their genetically related conventionalized counterparts, males and females, 6–8 weeks old, were used. Germfree animals were housed in groups of 3–6 in glass jar cages in sterile flexible film isolators (Trexler and Reynolds, 1957). They were supplied with sterilized tap water and Purina Chow diet (5010 C) *ad libitum*. Conventional mice were maintained in isolator units, but were occasionally exposed to the room environment during service. Water and diet were not sterilized.

Virus.—The Gledhill strain of mouse hepatitis virus (MHV-1) (Gledhill, Dick and Andrewes, 1952) was prepared from infected livers of suckling mice as described previously (Starr and Pollard, 1958). Fifty per cent infectious dose (ID_{50}) of virus was determined in suckling conventional mice and calculated according to the method of Reed and Muench (1938). Titres of stock virus ranged between $1 \times 10^{-3 \cdot 0}$ and $1 \times 10^{-4 \cdot 0}$ ID_{50} per 0·1 ml.

Determination of phagocytic index (K).—Phagocytic activity of the RES was measured by the carbon clearance method of Biozzi *et al.* (1953). Colloidal carbon was injected into the lateral tail vein at a dose of 16 mg. per 100 g. body weight. Animals were not anaesthetized. Blood samples were drawn from the orbital venous plexus at known times (min.) after inoculation of carbon and lysed in 2 ml. of 0·1 per cent sodium carbonate. Carbon concentration was determined spectrophotometrically. A straight line was obtained by plotting $\log_{10}$ carbon concentration against time in minutes. The slope of the line defines K, the phagocytic index. Experimental germfree mice were removed from their isolator and the test was performed during the next 30 min.

Triolein.—Glyceryl trioleate (practical grade, Sigma) was used as a 10 per cent emulsion as described by Stuart *et al.* (1960). One ml. triolein was mixed with 0·2 ml. Tween 20 and 8·8 ml. per cent aqueous glucose. The mixture was emulsified by shaking vigorously for 3–5 min. A fresh mixture was prepared on each day of use, and sterilized by filtration through a 0·45 μ Millipore filter. Depending upon the experimental procedure involved, mice were inoculated i.v. with a single injection of 0·1 ml., or a series of 2 i.p. injections of 0·4 ml., 24 or 48 hr apart. Where applicable, virus or cortisone plus virus followed triolein 48–72 hr later.

Cortisone.—Cortisone acetate (Upjohn) was administered i.p. (5·0 mg. per mouse) and was usually given 2 hr prior to virus where indicated.

Measurement of extent of macroscopic liver damage.—Comparative severity of infection following treatment of mice with combinations of triolein, cortisone, and virus was measured by visual observation of macroscopic lesions on the surface of the liver. Lesions were scored as moderate-to-severe if they involved 50–100 per cent of the liver surface, or slight if they involved no more than 25 per cent of the liver surface. Lesions were never found when mice were treated with triolein and/or cortisone in the absence of virus.

Statistical analysis.—Comparisons of extent of liver damage were analysed by the χ^2 test. Student's t test was used to analyse phagocytic indices.

RESULTS

Phagocytic activity after treatment with triolein, cortisone, and MHV-1

The phagocytic activity of the RES was measured by carbon clearance procedures as described above. Germfree and conventional mice, 7–8 weeks of age were inoculated with triolein, cortisone, or virus. Tiolein was administered in 2 doses of 0·4 ml., i.p., on consecutive days. Cortisone was given i.p. in a single dose of 5·0 mg. per mouse. A virus dose of 0·1 ml. undiluted stock MHV-1, representing 1×10^{-4} suckling mouse ID_{50} was inoculated i.p. The results in Table I show mean values for 4 or 5 animals. Untreated conventional mice had

TABLE I.—*Phagocytic Indices* (K *Values*) *for Germfree* (*GF*) *and Conventional* (*CONV*) *mice at Various Times after Treatment with Triolein, Cortisone and MHV*-1*

Time after inoculation (hr)	Triolein		Cortisone	Virus	Controls†	
	GF	CONV	GF	GF	GF	CONV
6	0·0117 (0·0011)		0·0058 (0·0027)		0·0117 (0·0018)	0·0165 (0·0010)
12	0·0094 (0·0021)		0·0028 (0·0008)	0·0110 (0·0024)		
24	0·0134 (0·0016)	0·0107 (0·0017)	0·0055 (0·0014)			
36	0·0181 (0·0020)		0·0074 (0·0017)	0·0125 (0·0018)		
48	0·0178 (0·0018)	0·0147 (0·0011)	0·0102 (0·0011)			
72	0·0165 (0·0024)	0·0164 (0·0014)	0·0100 (0·0009)	0·0145 (0·0020)		
144	0·0142 (0·0012)		0·0106 (0·0025)			

* *K* indices represent mean values for 4 or 5 mice. Standard deviation in parentheses.
† Controls were untreated.

higher K values (mean 0·0165) than untreated germfree mice (mean 0·0117). The difference was significant ($P < 0·05$). Triolein caused an initial depression of the K index of germfree and conventional mice. Following this, indices for germfree mice increased by 24 hr and remained elevated through 144 hr. There was no apparent hyperactivity of the RES in conventional mice during the observation period. A severe depression of K index was noted within 6 hr after cortisone treatment. Values returned to normal by 48 hr. Virus appeared to enhance the K index slightly over a 72 hr period. Control inocula of triolein carrier solution and normal liver suspension had no effect on the K index.

Effect of tiolein on severity of liver damage

The protective effect of triolein against a challenge of virus and also cortisone plus virus is shown in Table II. Two doses of triolein (0·4 ml.) preparation were administered i.p., 48 hr apart, to groups of 6-week-old germfree and conventional

TABLE II.—*Extent of Macroscopic Liver Damage in Germfree* (*GF*) *and Conventional* (*CONV*) *Mice after Treatment with Cortisone, Triolein and MHV*-1*

Group	Treatment	No. of mice	Per cent macroscopic liver lesions		
			None	Slight	Moderate-to-severe
1	V	15 GF	0	53	47
2	V	10 CONV	10	80	10
3	C/V	14 GF	0	29	71
4	C/V	7 CONV	0	29	71
5	T/C/V	10 GF	0	60	40
6	T/C/V	7 CONV	43	43	14
7	T/V	9 CONV	78	22	0

* Livers were scored 4 days after virus inoculation.

mice. Sixty hr after the second treatment, animals were challenged i.p. with virus or cortisone plus virus. A single dose of 5 mg. cortisone per mouse was given 1–2 hr prior to virus. Control mice included those treated with cortisone or triolein, alone. The extent of macroscopic liver damage was determined 4 days after virus challenge. Among conventional mice treated with virus alone (group 2), 80 per cent displayed slight liver involvement, 10 per cent moderate-to-severe liver involvement, and 10 per cent appeared normal. After triolein treatment (group 7), 78 per cent appeared normal. This was a significant protective effect ($P < 0{\cdot}01$). Tiolein treatment also resulted in protection of conventional mice treated with cortisone plus virus (groups 4 and 6). The percentage of moderate-to-severe infections decreased from 71 per cent in group 4 to 14 per cent in group 6 ($P < 0{\cdot}01$). Germfree mice (group 1) displayed more extensive liver involvement (47 per cent moderate-to-severe) after virus alone than did conventional mice (group 2) ($P < 0{\cdot}05$). The percentage of moderate-to-severe infections in germfree mice increased to 71 per cent when cortisone accompanied virus (group 3). Among cortisone-treated germfree mice, the reduction in severity of infection following triolein pretreatment was not significant (groups 3 and 5). Thus, triolein reduced the deleterious action of virus and also that of cortisone plus virus among groups of germfree and conventional mice when both triolein and virus were administered i.p., although protection of cortisone-treated germfree mice by triolein was slight.

Attempts were made to demonstrate a protective effect of triolein on virus administered i.v. Conventional mice were pretreated with either a single i.v. dose of triolein or 2 i.p. doses on consecutive days. Forty-eight hr after triolein treatment, animals were challenged either i.v. with 1×10^{-3} suckling mouse ID_{50} or i.p. with 1×10^{-4} ID_{50} of MHV-1. Inoculations i.v. were performed *via* the lateral tail vein without anaesthesia. Protocol and results are given in Table III.

TABLE III.—*Effect of Route of Inoculation on Extent of Liver Damage in Conventional Mice after Treatment with Triolein amd MHV-1*

Route				Per cent macroscopic liver lesions		
Group	Triolein	Virus	No. Mice	None	Slight	Moderate-to-severe
1	—	i.p.	6	33	66	0
2	i.p.	i.p.	6	100	0	0
3	—	i.v.	6	0	15	85
4	i.p.	i.v.	6	0	0	100
5	i.v.	i.v.	6	0	15	85

Mice treated with virus alone and triolein plus virus by the i.p. route (groups 1 and 2) again demonstrated the protective effect of triolein. However, when mice were pretreated with triolein, either i.p. (group 4) or i.v. (group 5), and challenged with virus i.v., no protective effect was seen when compared to virus alone (group 3). It appears from the results of this and the preceding experiment that pretreatment of mice with triolein provided protection against the severe effects of virus alone or cortisone plus virus when challenged i.p. but not i.v.

DISCUSSION

Quantitatively, liver macrophages (Kupffer cells) are the most important cells involved in clearance of foreign material, including viruses, from the blood (Mims,

1964). With respect to the MHV viruses, these cells assume additional importance since they support the growth of virus and may thereby contribute to the development of liver infection. The question has been raised whether the pathogenicity of the hepatotrophic virus is related to the phagocytic activity of the RES. Gledhill, Dick and Niven (1965*a*) and Gledhill, Bilbey and Niven (1965*b*) found that *Eperythrozoon coccoides* and Friend and Maloney leukaemia viruses, agents which enhance the pathogenicity of MHV-1, also stimulated the RES as measured by carbon clearance. However, stilboestrol, which also enhanced the clearance of carbon, had no effect on the pathogenicity of MHV-1. In our studies, we used triolein and cortisone, substances which, respectively, stimulated and suppressed RES clearance activity. At the same time, triolein was shown to increase resistance against an i.p. challenge of virus, while cortisone greatly decreased resistance.

Triolein (glyceryl trioleate) is a simple triglyceride whose effect on RES function *in vivo* and on phagocytosis *in vitro* was demonstrated by Stuart *et al.* (1960) and Cooper (1964). Furthermore, triolein has been reported to selectively stimulate only fixed macrophages when inoculated i.v. (Cooper and Stuart, 1961). We found that an i.p. inoculation of triolein moderately stimulated clearance of carbon in germfree mice but had no significant effect in conventional mice. Nevertheless, i.p. administration of triolein resulted in significant protection of both germfree and conventional mice when the route of MHV-1 challenge was also i.p. If protection by triolein was associated with stimulation of liver macrophages, one would expect to find increased resistance to an i.v. challenge of virus. Such was not the case. After i.p. injection, it is unlikely that triolein reaches the liver in adequate amounts of the proper droplet size to exert optimal effect on liver macrophages. However, when triolein was administered i.v., it again failed to protect against an i.v. challenge of virus. It would appear, therefore, that the protective effect of triolein was exerted elsewhere than in the liver. It is important to recall that MHV viruses multiply in peritoneal macrophages (Kantoch, Warwick and Bang, 1963), and that following i.p. inoculation of MHV-1 effects of virus are seen in mesothelial linings of the peritoneum (Gledhill *et al.*, 1955*a*, *b*). Since triolein is known to be taken up by macrophages *in vitro* and to enahance particle uptake by these cells (Cooper, 1964), it is possible that its protective action is exerted locally in the area of the peritoneum which is the site of inoculation.

Cortisone greatly suppressed RES function and was seen to decrease resistance of both germfree and conventional mice to MHV-1 as measured by severity of liver lesions (Table II). These results are consistent with the general view that impairment of RES function is associated with decreased resistance to infectious agents. In previous experiments with cortisone-treated mice (Lavelle and Starr, 1968*b*), the effect of RES suppression on early virus titres was examined. It was found that virus titres of liver 24 hr after i.v. challenge of MHV-1 were similar in cortisone-treated and untreated mice. Thus, cortisone did not appear to alter the infectivity of MHV-1. Rather, the effect of the steroid was to prolong virus production and thereby increase final yields, possibly by suppression of interferon formation.

When triolein treatment preceded combined cortisone-virus challenge, significant protection of conventional mice was seen, but only slight protection of germfree mice was observed. This observation suggests that conventional mice may have been more responsive than germfree mice to the protective effects of

triolein. However, conventional mice, as shown in Table I, were less responsive than germfree mice to the stimulating effects of triolein on carbon clearance. These results support the contention that increased resistance to MHV-1 following triolein treatment was not related to the carbon clearing activity of the RES.

REFERENCES

BANG, F. B. AND WARWICK, A.—(1960) *Proc. nat. Acad. Sci.*, **46**, 1065.
BIOZZI, G., BENACERRAF, B. AND HALPERN, B. N.—(1953) *Br. J. exp. Path.*, **34**, 441.
—(1955) *Br. J. exp. Path.*, **36**, 226.
COOPER, G. N.—(1964) *J. reticuloendothel. Soc.*, **1**, 50.
COOPER, G. N. AND STUART, A. E.—(1961) *Nature, Lond.*, **191**, 294.
GLEDHILL, A. W., BILBEY, D. L. J. AND NIVEN, J. S. F.—(1965) *Br. J. exp. Path.*, **46**, 433.
GLEDHILL, A. W., DICK, G. W. A. AND ANDREWES, C. H.—(1952) *Lancet*, ii, 509.
GLEDHILL, A. W., DICK, G. W. A. AND NIVEN, J. S. F.—(1955) *J. Path. Bact.*, **69**, 299.
KANTOCH, M., WARWICK, A. AND BANG, F. B.—(1963) *J. exp. Med.*, **117**, 781.
LAVELLE, G. C. AND STARR, T. J.—(1968*a*) *J. reticuloendothel. Soc.*, **5**, 422.—(1968*b*) *J. reticuloendothel. Soc.*, **5**, 436.
MIMS, C. A.—(1964) *Bact. Rev.*, **28**, 30.
REED, L. J. AND MUENCH, H.—(1938) *Am. J. Hyg.*, **27**, 493.
RUEBNER, B. AND MIYAI, K.—(1962) *Am. J. Path.*, **40**, 425.
STARR, T. J. AND POLLARD, M.—(1958) *Proc. Soc. exp. Biol. Med.*, **99**, 108.
STUART, A. E., BIOZZI, G., STIFFEL, C., HALPERN, B. N. AND MOUTON, D.—(1960) *Br. J. exp. Path.*, **41**, 599.
TREXLER, P. C. AND REYNOLDS, L. I.—(1957) *Appl. Microbiol.*, **5**, 406.
VELLA, P. P. AND STARR, T. J.—(1965) *J. infect. Dis.*, **115**, 271.