Clin. exp. Immunol. (1977) **29**, 147–151.

Cellular immune responses to amoebic liver abcess in the guinea-pig

R. S. BRAY & W. G. HARRIS *Medical Research Council Laboratories, Fajara, The Gambia, West Africa*

(*Received* 31 *January* 1977)

SUMMARY

Guinea-pigs infected in the liver with the Biswas strain of *Entamoeba histolytica* showed no dermal hypersensitivity but showed positive lymphocyte transformation and macrophage-migration inhibition.

The time sequence showed an activated response at 4 days after infection, a full response at 8 days when the liver abscesses were resolving and a waning response at 12 days when the abscesses had healed.

INTRODUCTION

Previous studies have shown that, in man, cellular immune responses may play a significant role in limiting the extent and severity of infection with *Entamoeba histolytica* in the field and that development of clinical hepatic or intestinal amoebiasis is preceded by, and associated with, significant specific cellular immunodepression (Harris & Bray, 1976, 1977). These studies were designed to investigate further possible defects in cellular immune response in experimental amoebiasis.

Experiments with animal models of amoebic infection have been concerned primarily with the elucidation of pathological mechanisms (Reinertson & Thompson, 1951; Gogler & Knight, 1974), the differentiation of pathogenic and non-pathogenic strains of parasite (Neal & Vincent, 1955; Healy & Gleason, 1966), or the investigation of potential new anti-amoebic drugs (Singh, Das & Saxena, 1963). Very little significant work appears to have been published on the immune response to experimental infection with *E. histolytica* in laboratory animal species.

This paper reports the results of studies on the short-term cellular immune responses of the guinea-pig to experimental infection due to *E. histolytica*.

MATERIALS AND METHODS

The parasite. E. histolytica of the known pathogenic Biswas strain was obtained through the courtesy of Dr R. A. Neal of the Wellcome Foundation Ltd, Beckenham, and maintained locally on HSre slopes with rice starch (Dobell & Laidlaw, 1926). For inoculation, the rice starch deposit from a number of 48-hr culture bottles was pooled and washed three times with Ringers–egg albumin by centrifugation at 900 rev/min for 10 min. A 1/10 dilution of the sediment was counted and the number of organisms in the final preparation adjusted to 5×10^5/ml.

Infection procedure. Colonic infection of young, locally reared, male, inbred WAG rats (ex-Medical Research Council Laboratory Animals Centre, Carshalton, England) was attempted by laparotomy and direct intracaecal inoculation of 0·2 ml of the suspension containing 10^5 organisms (Neal, 1951).

Hepatic infections of locally reared Hartley guinea-pigs were established by direct inoculation without laparotomy. A 21-gauge needle was introduced into the peritoneal cavity immediately alongside the xyphoid cartilage. The point of the needle was advanced dorsally into the liver tissue and 0·2 ml of the suspension containing 10^5 organisms was injected.

Guinea-pigs. Blood was collected by cardiac puncture under ether anaesthesia 4, 8 and 12 days after infection and added to heparinized tubes to give 20 iu/ml of anti-coagulant. The lymphocytes were prepared on a Ficoll–Hypaque gradient, and cultured in a microplate system, labelled with [^{3}H]thymidine and harvested as has been previously described for human cells

Correspondence: Dr R. S. Bray, MRC Laboratories, Fajara, The Gambia, West Africa.

(Harris & Bray, 1976). Stimulants were 1 μg, 10 μg or 50 μg of amoebic antigen. Counts per minute were transformed to $\log_{10}$ for statistical analysis. Appropriate cultures of normal cells with antigen were included to control any non-specific mitogenic effect of the antigen.

Similar tests were performed on the intracaecally infected rats.

Macrophage-migration inhibition assay. Starch-induced peritoneal exudate (PE) cells from guinea-pigs infected 4, 8 and 12 days previously were allowed to migrate from the cut ends of 100 λ capillary tubes for 24 hr into 10% foetal calf serum-medium 199 containing 0·1 or 10 μg amoebic antigen/ml. Inhibition of macrophage migration was expressed as a migration ratio. Similar tests were performed on the intracaecally infected rats.

Skin tests. Pairs of guinea-pigs were skin tested at 4, 8 and 12 days post-infection with 0·1 μg, 1·0 μg and 10·0 μg of antigen in 0·1 ml of saline, together with a saline control. Animals were shaved with electric clippers and depilated before inoculation. Skin thickness of the marked injection sites were recorded at 30 min, and at 24, 48 and 72 hr after injection.

Transfer of skin sensitivity by cells. The spleens and PE cells excited by 20 ml of sterile paraffin oil inoculated 4 days previously were removed from sixteen guinea-pigs with 12-day-old amoebic liver infections.

The pooled PE cells were washed four times and inoculated i.p. into two guinea-pigs. The spleens were teased in medium 199, forced through a stainless-steel sieve (80 mesh/in) and passed down a column of ballotini beads at 37°C. The effluent suspension was washed four times and inoculated i.p. into two guinea-pigs. Immediately after inoculation each recipient guinea-pig was skin tested by the i.d. inoculation of saline, 100 μg and 500 μg amoebic antigen.

Lymphokine studies. PE cells, spleen cells and peripheral blood lymphocytes were collected into TCM 199 from four guinea-pigs 12 days after liver infection. The PE cells and spleen cells were pooled and the two cell suspensions were washed, adjusted to 10^7 cells/ml and each divided into two equal aliquots. Amoebic antigen, 10 μg/ml, was added to one aliquot of each suspension and the aliquots were then incubated at 37°C for 48 hr, after which the cells were spun down and the supernatants collected. The aliquots which had not previously received antigen were dosed with 10 μg/ml of antigen and employed as controls. The supernatants were tested for mitogenic activity in guinea-pig lymphocyte cultures.

Serology. Specific anti-amoebic whole immunoglobulin titres were determined by the indirect fluorescent antibody method of Kane, Matossian & Batty (1971), using anti-species specific fluorescent conjugates (Wellcome Reagents Ltd, Beckenham, England).

RESULTS

Rats

Groups of three animals were autopsied 4, 8 and 12 days after infection. At day 4, one of the three had amoebae in the caecal contents that were morphologically indistinguishable from *E. histolytica*, but no evidence of pathology applying the criteria of Neal (1951). No amoebae were seen in the other two animals, and no organisms were isolated by culture of caecal contents. At days 8 and 12 no animals showed evidence of pathology or infection at autopsy.

No specific lymphocyte transformation and no significant macrophage-migration inhibition were detected, although cells did respond *in vitro* to PHA. No parasite-specific circulating antibody was detected at any stage.

Guinea-pigs

Groups of four guinea-pigs were bled out and autopsied at 4, 8 and 12 days post-infection.

At day 4, single or multiple liver abscesses were found in all guinea-pigs, varying in total volume from below 1% to approximately 15% of the liver tissue, with in three animals quantities of green-yellow flocculent pus and adhesions between liver and diaphragm, or between liver and the different parts of the bowel or mesenteries. Amoebae were identified by direct microscopic examination of the normal liver tissue taken from just beyond the periphery of the lesion or by culture of similar samples.

Bacterial infection of the abscess was inevitable under these experimental conditions, as the culture medium from which the inoculum was prepared contains a mixed flora essential to the *in vitro* growth of this strain of amoebae, and probably also to the expression of maximum pathogenic potential (Phillips, Wolfe & Bartgis, 1958; Neal, 1971). The use of antibiotics was avoided as this may substantially reduce the success of the invading amoebae. The majority of deaths occurred within 72 hr of inoculation, and appeared to be due in all animals examined *post-mortem* to acute peritonitis rather than to a progressive liver infection.

At day 8 abscesses were found in all animals examined but these were smaller—from below 1% to approximately 5% of the liver tissue—and amoebae were only positively identified in one of the four

animals autopsied at this time interval. Pus and adhesions were still prominent features of the pathology, and in one animal an amoebic pericarditis was noted.

At day 12 two of the animals examined had small healed lesions identified by scarring of the liver tissue and persistent adhesions in one case. The other two animals had small, apparently resolving abscesses of below 1% of the liver tissue in volume. No amoebae were detected by direct examination or culture in these animals.

Immune parameters from these animals are summarized in Fig. 1. At day 4 a positive lymphocyte-

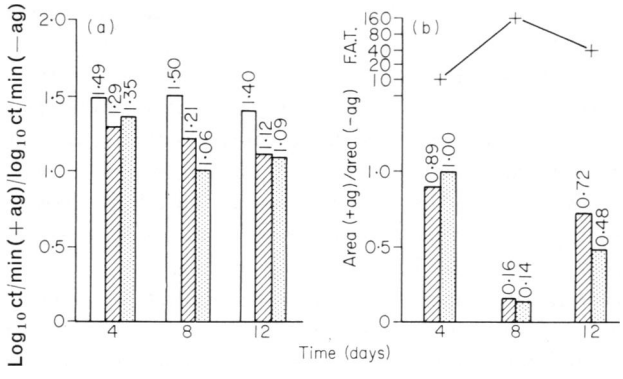

FIG. 1. Immune parameters of the guinea-pig following hepatic infection with *Entamoeba histolytica*. (□) PHA; ▨, AA1 = 1 μg amoebic antigen; ⊡ AA10 = 10 μg amoebic antigen. (a) Lymphocyte transformation; (b) macrophage-migration inhibition.

transformation response was detected, but associated with a negative macrophage-migration inhibition assay. At day 8, the transformation response was positive, although the level of thymidine uptake was slightly less than at day 4. The direct MIF assay was strongly positive at this time, giving area ratios between experimental and control of 0·16 with 1 μg/ml antigen and 0·14 with 10 μg/ml antigen. At day 12, the lymphocyte response to amoebic antigen was detectable, but very much less than at days 4 and 8. The MIF response was still markedly positive, although less so than at day 8.

The specific anti-amoebic immunoglobulin titre varied from 1/10 at day 4, to 1/160 at day 8 and 1/40 at day 12.

Cells from uninfected animals gave negative responses at all dose levels of antigen employed.

Direct skin tests were negative at days 4, 8 and 12 at all antigen dose levels; equally negative skin tests were obtained following cell transfers.

The supernatants from the antigen-stimulated peripheral blood lymphocytes showed no mitogenic activity. The ratio of the log_{10} ct/min stimulated : control was 1·04 at concentration 1 in 1 and 1·00 at concentration 1 in 5. The supernatants from the antigen-stimulated PE and spleen cells were highly active. The ratio of the log_{10} ct/min stimulated : control was 1·48 at concentration 1 in 1 and 1·69 at concentration 1 in 5.

DISCUSSION

Previous studies have shown that the rat is susceptible to amoebic infection of the colon for only a very short period of time at the age of weaning and that susceptibility is lost very rapidly with increasing age, although an established infection may persist for several months with continuing pathology (Neal, 1951). Successful colonic infections in rats were not established in this study, the rats used being 6–10 weeks of age and presumably outside the period of susceptibility. Our data suggest that specific immune mechanisms are not involved in the prevention of infection in these animals.

Liver abscesses were successfully established in young adult guinea-pigs by direct inoculation of amoebae plus associated bacteria into the liver tissue. The high mortality occurring in the first 72 hr

was associated with bacterial peritonitis rather than hepatic amoebiasis, although a few deaths did occur after day 3 and these were associated with large liver lesions.

The immune parameters summarized in Fig. 2 may be interpreted in at least two ways. The positive lymphocyte transformation at day 4 represents the induction phase of the immune response with, presumably, at this stage an increasing population of peripheral sensitized cells. Over the next 8 days, the drop in lymphocyte-transformation response could indicate an active immunodepression, a suggestion supported by the reduction in the positive MIF response at day 12, and the drop in antibody titre.

However, a more likely interpretation is that these parameters are indicating the successful response of the host against the invading amoebae. At day 4, a positive lymphocyte-transformation response was seen with a negative MIF—this was the inductive phase of immunity. At day 8, a lower lymphocyte-transformation response with a very high MIF response was seen—this was the effective phase of immunity. At day 12, both responses were positive, but reduced—this was the recovery phase. Antibody levels followed a similar pattern—negative on day 4 during induction, highest on day 8 during the effector phase, and raised but lower on day 12 during recovery. This hypothesis was supported by the parasitological evidence; in general, active lesions were seen on day 4, active and resolving lesions on day 8, and resolving lesions only on day 12 in surviving animals.

Direct skin testing of animals at all stages post-infection gave negative results. The correlation between the manifestations *in vivo* of delayed hypersensitivity and the *in vitro* correlates is far from exact, with generally *in vivo* effects developing later than the more sensitive *in vitro* correlates. Given the positive *in vitro* results, a positive *in vivo* skin test reaction would be expected with time.

Ortiz-Ortiz *et al.* (1973) have reported the appearance of MIF responses in the hamster 5 days post-infection, with disappearance at day 10–20, and reappearance at day 25. In the guinea-pig, the MIF response was negative at day 4, positive at day 8 and disappearing at day 12. Examination at a later time interval may show the reappearance of the MIF response, with possibly *in vivo* manifestations also appearing at this time.

How may these results be correlated with the field situation? Clearly the response to an infection established directly in the liver tissue does not in any way parallel what may be happening in the field. The animals will not be immunodepressed by a pre-existing gut infection, or by other environmental factors, and the response generated will be a classical response to an injected antigen or organism. Any ability of the amoebae to depress the efferent arm of the immune response may perhaps be studied in this system but the influence of clinical or sub-clinical intestinal infection on the efferent arm cannot be determined with this model.

Maegraith & Harinasuta (1954a,b) have described a method for inoculating amoebae into the liver of guinea-pigs via the mesenteric and portal veins—a procedure that may resemble more closely the real-life situation. However, the liver lesions produced were rapidly resolved and the authors suggested that either damage or 'sensitization' from a pre-existing gut infection may be required for development of a progressive liver lesion. Perhaps what is in fact required is not sensitization, but immunodepression due to an existing gut infection for establishment of a liver lesion. Physical damage certainly does appear to render the healthy liver susceptible to infection (Gogler & Knight, 1974), but in our experience liver abscesses in man were most frequently seen in basically healthy young adults in whom predisposing damage to the liver tissue would be minimal.

Intraperitoneal inoculation of amoebae in hamsters, but not in guinea-pigs, may also lead to liver infection (Jarumilinta, 1966), although again this situation does not parallel closely what may be occurring in the field. What is required is a method for establishing gut infections in an experimental host that may subsequently lead to a liver infection via the portal vein. This may require the investigation of various experimental procedures including treatment with immunodepressives, but would allow the closer study of the immune status immediately prior to, as well as during, both liver and gut infections. This would appear to be the critical area of research at the present time.

The valuable technical assistance of Mr J. Brown and Mr M. A. Bray is acknowledged.

REFERENCES

DOBELL, C. & LAIDLAW, P.P. (1926) On the cultivation of *Entamoeba histolytica* and some other entozoic amoebae. *Parasitology*, **18**, 283.

GOGLER, H. & KNIGHT, R. (1974) The effect of hepatic injury upon the development of amoebic liver abscess in hamsters. *Ann. trop. Med. Parasit.* **68**, 177.

HARRIS, W.G. & BRAY, R.S. (1976) Cellular sensitivity in amoebiasis—preliminary results of lymphocyte transformation to specific antigen and PHA in carrier and disease states. *Trans. roy. Soc. trop. Med. Hyg.* **70**, 340.

HARRIS, W.G. & BRAY, R.S. (1977) The epidemiology of amoebiasis in The Gambia, West Africa. *Trans. roy. Soc. trop. Med. Hyg.*

HEALY, G.R. & GLEASON, N.N. (1966) Studies on the pathogenicity of various strains of *Entamoeba histolytica* after prolonged cultivation, with observations on strain differences in the rats employed. *Am. J. trop. Med. Hyg.* **15**, 294.

JARUMILINTA, R. (1966) A simple method for inducing amoebic liver abscess in hamsters. *Ann. trop. Med. Parasit.* **60**, 139.

KANE, G.J., MATOSSIAN, R. & BATTY, I. (1971) Fluorochrome-labelled anti-immunoglobulin fractions used with stabilized antigen preparations for the assessment of parasitic diseases. *Ann. N.Y. Acad. Sci.* **177**, 134.

MAEGRAITH, B.G. & HARINASUTA, C. (1954a) Experimental amoebic infection of the liver in guinea pigs. I. Infection via the mesenteric vein and via the portal vein. *Ann. trop. Med. Parasit.* **48**, 421.

MAEGRAITH, B.G. & HARINASUTA, C. (1954b) Experimental amoebic infection of the liver in guinea pigs. II. Abscess formation with persistent intestinal lesions. *Ann. trop. Med. Parasit.* **48**, 434.

NEAL, R.A. (1951) The duration and epidemiological significance of *Entamoeba histolytica* infections in rats. *Trans. roy. Soc. trop. Med. Hyg.* **45**, 363.

NEAL, R.A. (1971) Pathogenesis of amoebiasis. *Gut*, **12**, 483.

NEAL, R.A. & VINCENT, PATRICIA (1955) Strain variation in *Entamoeba histolytica*. I. Correlation of invasiveness in rats with the clinical history and treatment of the experimental infections. *Parasitology*, **45**, 152.

ORITZ-ORTIZ, L., ZAMACONA-RAVELO, G. & CAPIN, N.R. (1974) Hipersensibilidad celular en amibiasis. III. Efecto *in vitro* de la concanavalina A y de antígeno amibiano sobre leucocitos periféricos de pacientes con absceso hepático amibano. *Arch. Invest. Med.* (*Mex.*) **5**, supplement 2, 481.

PHILLIPS, B.C., WOLFE, PATRICIA, A., & BARTGIS, DA L. (1958) Studies of the ameba-bacteria relationship in amebiasis. II. Some concepts on the etiology of the disease. *Am. J. trop. Med. Hyg.* **7**, 392.

REINERTSON, J.W. & THOMPSON, P.E. (1951) Experimental amebic infection in hamsters. *Proc. Soc. exp. Biol.* (*N.Y.*), **76**, 518.

SINGH, B.N., DAS, S.R. & SAXENA, USHA (1963) Virulence of strains of *Entamoeba histolytica* from India, with an account of a method for obtaining 100 per cent infection in rats. *Ann. Biochem. exp. Med.* **23**, 237.