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Survival of bacteria in amoebic hepatic pus *in vitro*

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INTRODUCTION

One characteristic feature of hepatic amoebiasis with abscess formation is the finding that pus obtained by needle aspiration of the liver is usually sterile when cultured for bacteria, although amoebae may be found readily. The review article by DeBakey & Ochsner (1951), for example, quotes an incidence of 87·7 % of sterile cultures from 864 samples examined. Lamont & Pooler (1958) found 88·6 % sterile cultures from 106 specimens examined and Sepulveda, Jinick, Bassols & Munoz (1959) 81·3 % from eighty samples. However, it is not known whether any of these findings apply to primary and/or secondary aspiration specimens. A comparative investigation of this type carried out by Maddison, Powell & Elsdon-Dew (1959), revealed seventy-three sterile specimens from seventy-five primary aspirations, the remaining two yielding cultures of *Aerobacter aerogenes*. Twenty-nine patients in this series were subjected to aspiration a second time, six additional positive cultures being obtained. Scragg (1960), on the other hand, found thirty-six of forty (90 %) primary aspirations to be bacteriologically sterile, but in no patient in her series of African children was aspiration followed by secondary bacterial infection.

Table 1. *Nature of organisms isolated from amoebic hepatic pus*

(No. of specimens examined, 289; no. of positive isolations, 58.)

Organisms	No. of isolations	Organisms	No. of isolations
Escherichia coli	17	*Staphylococcus saprophyticus*	4
Aerobacter aerogenes	3	Non-haemolytic streptococci	3
Proteus mirabilis	2	*Esch. coli* and *Proteus mirabilis*	4
Pseudomonas aeruginosa	1	*Esch. coli* and *A. aerogenes*	1
Salmonella species	5	*Proteus vulgaris* and paracolon species	1
Bacillus alkaligenes	1	*Esch. coli* and non-haemolytic streptococci	2
Paracolon species	2	*Esch. coli* and *Ps. aeruginosa*	1
Staphylococcus pyogenes	10	*Esch. coli*, *A. aerogenes* and *Proteus mirabilis*	1

On *a priori* grounds one might expect to find a high incidence of bacterial infection in amoebic hepatic abscess since it would appear to be a reasonable assumption that intestinal organisms as well as amoebae, can penetrate the damaged bowel mucosa and reach the liver via the portal radicles, either in association with amoebae or independently. The types of organisms listed in Table 1 indicate the gut as being the most likely source of their origin. These were cultured

from 289 patients with amoebic hepatic abscess admitted to King Edward VIII Hospital, Durban, during the past 4 years, and on whom aspiration was performed. The incidence of sterile samples in this series is 79·6 %. The failure to find organisms in the majority of amoebic hepatic abscesses suggested that liver pus might exert an inhibitory action on a number of bacteria. Some *in vitro* investigations of this nature are presented here.

MATERIAL AND METHODS

Amoebic liver pus

Liver pus was obtained from patients by needle aspiration of the enlarged viscus, and was stored in sterile 30 ml. screw-cap containers at 4·0° C. The amoebic origin of the pus was confirmed by both direct demonstration and culture of *Entamoeba histolytica* from each specimen. Gram-stained films did not reveal the presence of bacteria and routine bacteriological cultures, both aerobic and anaerobic, were sterile. Experimental investigations on liver pus were carried out as soon as possible and no specimen was kept for more than 14 days.

Bacterial cultures

Inocula were prepared from the following strains: *Escherichia coli* 5396/38, *Staphylococcus pyogenes* N.C.T.C. 6571, *Salmonella typhi* strain O 901, and freshly isolated local strains of *Streptococcus faecalis, Aerobacter aerogenes, Shigella sonnei, Shigella boydii, Salmonella typhimurium* and an organism of pseudomonas species.

Cultures were maintained in Robertson's cooked meat medium. Single colonies from subcultures on nutrient agar (Difco), were inoculated into nutrient digest broth and subcultures in broth were transferred daily for ten days. Inocula were prepared from serial tenfold dilutions of broth culture in sterile phosphate buffered saline, the number of viable organisms being determined by surface plate counts (Miles & Misra, 1938).

Complement

Complement consisted of pooled guinea-pig serum obtained by cardiac puncture and pooled after overnight separation at 4·0° C. Prior to use, fractions of serum were absorbed on two occasions, for 1 hr. each, with heavy heat killed suspensions of the appropriate strains to remove any naturally occurring bactericidal factors.

Streptodornase solution

With each experimental procedure carried out a parallel set of tests was included, incorporating streptodornase solution (500 units in 0·1 ml. volume) in the form of 'Varidase' (Lederle). This was done to overcome the possibility of uneven mixing of bacterial inoculum and liver pus due to the viscid nature of the latter. Streptodornase produces a rapid decrease in viscosity of liver pus and preliminary experiments showed that the concentration employed did not affect the viability of any of the inoculum strains. No differences were observed in the results from the two series of tests.

EXPERIMENTS AND RESULTS

The majority of tests were carried out in replicate series of sterile 3 in. by $\frac{1}{2}$ in. tubes by mixing 1·0 ml. of liver pus with 0·1 ml. of bacterial inoculum and 0·1 ml. of either sterile saline or streptodornase solution. Where the effect of complement was being investigated, this was added in 0·1 ml. aliquot amounts of undiluted absorbed guinea-pig serum. All tubes were incubated at 37·0° C. under appropriate atmospheric conditions. At intervals of time up to 5 days, aliquot amounts were removed and surface viable counts performed as described.

Survival of different organisms in liver pus

The results detailed in Table 2 show the effect of liver pus on a number of different types of bacteria. In each instance the initial inoculum consisted of an appropriate dilution of a 12 hr. broth culture in phosphate buffered saline. The tubes were incubated in an aerobic atmosphere.

A variable response was observed with different organisms. The strain of *Staph. pyogenes* was completely inhibited between the fifth and twelfth hour of incubation and both the strain of *Sh. sonnei* and pseudomonas species were inhibited in less than 5 hr. With the exception of *A. aerogenes* the remaining organisms were subjected to an initial fall in viable count during the first few hours of incubation followed by a logarithmic increase in numbers. Table 2 also shows

Table 2. *Effect of liver pus on various organisms*

Organism	Inoculum: no. of organisms at zero time	Bacterial count/ml. after incubation for				
		2 hr.	5 hr.	12 hr.	24 hr.	5 days
Esch. coli	200	< 10	100	2×10^8	10^8	10^5
Salm. typhimurium	220	20	320	10^8	4×10^7	10^6
Sh. sonnei	180	< 10	Nil	Nil	Nil	Nil
Staph. pyogenes	900	800	550	Nil	Nil	Nil
Strep. faecalis	500	52	480	10^5	8×10^4	10^3
Salm. typhi	2×10^3	$1{\cdot}1 \times 10^3$	10^4	10^8	3×10^7	2×10^5
Sh. boydii	10^3	10^3	800	2×10^5	10^5	3×10^3
A. aerogenes	10^3	900	2×10^4	2×10^8	8×10^7	3×10^5
Pseudomonas species	600	120	Nil	Nil	Nil	Nil

Initial inoculum—dilution of 12 hr. broth culture. Control broth cultures gave viable counts, after 12 hr. incubation of between 10^8 and 10^9 organisms for each strain.

that *Esch. coli*, *Salm. typhimurium*, *Salm. typhi* and *A. aerogenes* attained to a maximum viable population census which was practically identical to that found with control cultures of the same organisms in nutrient broth, the only differences in fact being the initial killing of a certain percentage of the inocula in liver pus and a prolongation of the lag phase. Both *Strep. faecalis* and *Sh. boydii*, on the other hand, although showing a rapid increase in numbers after the early fall, were subjected to some form of inhibition and reached a maximum population density of only some 2×10^5 organisms/ml. Essentially similar results were obtained in replicate tests conducted under anaerobic conditions of growth.

Effect of age of inoculum

A series of standardized inocula prepared from broth cultures of varying ages, were added to liver pus as described. Bacterial counts performed at different times of incubation are shown in Table 3 for four of the strains.

Young cultures of both *Sh. sonnei* and *Staph. pyogenes* (12 hr. or less), were rendered sterile in liver pus in from 2 to 5 hr. Older cultures (24–36 hr.) of comparable inoculum size, again showed an initial fall in viable count during the first few hours of incubation followed by a logarithmic increase in numbers. However, both of these strains were inhibited, in the same manner as was observed with *Sh. boydii* and *Strep. faecalis*, from reaching the maximum population density which they attained to in control broth cultures. Young cultures of *Esch. coli* and

Table 3. *Effect of age of culture on survival of organisms in liver pus*

Organism	Age of culture (hr.)	Inoculum: no. of organisms at zero time	Bacterial count/ml. after incubation for 2 hr.	5 hr.	24 hr.	5 days
Esch. coli	5	200	< 10	80	6×10^8	3×10^5
	12	180	< 10	220	8×10^8	4×10^5
	24	150	200	450	10^9	10^6
	36	250	220	600	8×10^8	2×10^6
Salm. typhimurium	5	300	20	180	7×10^8	10^5
	12	150	10	200	6×10^8	2×10^5
	24	220	180	300	8×10^8	4×10^5
	36	300	200	280	10^9	10^5
Sh. sonnei	5	200	< 10	Nil	Nil	Nil
	12	100	15	Nil	Nil	Nil
	24	160	100	10^3	2×10^6	3×10^5
	36	120	120	10^3	3×10^3	10^5
Staph. pyogenes	5	180	< 10	Nil	Nil	Nil
	12	140	< 10	Nil	Nil	Nil
	24	220	150	2×10^3	10^7	4×10^5
	36	120	100	6×10^3	3×10^7	4×10^5

Salm. typhimurium also showed an initial drop in viable count during the initial stages of incubation but sufficient survivors remained to produce counts of the order of 10^8–10^9 organisms/ml. after 12 hr. incubation. Older cultures of *Esch. coli* and *Salm. typhimurium*, on the other hand, were not subjected to an initial fall in numbers, there being merely a prolongation of the lag phase followed by logarithmic increase in numbers. It appears that young cultures are more susceptible to the presence of certain inhibitory factors in liver pus than are older cultures. With some organisms, however, this inhibition is not sufficiently great to cause complete sterility (except perhaps with very small inocula), and the survivors are enabled to grow. The failure of some organisms to reach a maximum population census similar to control broth cultures, may represent either an inhibiting effect of the liver pus or some form of adaptive response on the part of the organism to the particular environment.

Effect of inoculum size

In Table 4 are detailed representative results of a number of investigations carried out with inocula of varying sizes.

Inocula of *Sh. sonnei* of from 50 to 500 organisms were destroyed in less than 5 hr. With a larger inoculum (5×10^3 organisms), the initial fall in viable count was followed by an increase in numbers to a population of 2×10^7 organisms after 12 hr. *Staph. pyogenes* behaved in a similar manner but both strains showed a reduction from their maximal attainable populations in broth. The survival of even small inocula of *Esch. coli* and *Salm. typhimurium* is again illustrated, and in both cases the lag phase was shorter the larger the inoculum size. *Sh. boydii* behaved like *Esch. coli* in the first few hours of incubation but again the maximum population, even with the highest inoculum, was only 4×10^5 organisms.

Table 4. *Effect of varying inocula on the bactericidal activity of liver pus*

Organism	Inoculum: no. of organisms at zero time	Bacterial count/ml. after incubation for 2 hr.	5 hr.	12 hr.	24 hr.	5 days
Esch. coli	10	Nil	12	10^8	8×10^7	4×10^5
	100	10	86	3×10^8	7×10^7	4×10^5
	1000	900	2×10^4	4×10^8	10^8	3×10^5
Sh. sonnei	50	Nil	Nil	Nil	Nil	Nil
	500	36	Nil	Nil	Nil	Nil
	5000	180	200	2×10^7	10^7	10^3
Salm. typhimurium	24	Nil	8	2×10^8	10^8	3×10^5
	240	76	400	4×10^8	2×10^8	2×10^5
	2400	3×10^3	5×10^4	4×10^8	3×10^8	10^5
Sh. boydii	42	2	24	10^5	2×10^5	10^3
	420	12	180	10^5	2×10^5	3×10^3
	4200	5×10^3	2×10^4	3×10^5	$1{\cdot}5 \times 10^5$	2×10^3
Staph. pyogenes	70	< 10	Nil	Nil	Nil	Nil
	700	160	4	Nil	Nil	Nil
	7000	560	780	2×10^7	8×10^6	10^4

Inocula prepared from 12 hr. broth culture.

Effect of complement

Complement in 0·1 ml. amounts was added to 1·0 ml. of liver pus together with 0·1 ml. of a suitable dilution of an overnight broth culture. Control tubes containing buffered saline in place of liver pus and complement respectively were included. Viable counts following incubation are shown in Table 5. for *Esch. coli* and *A. aerogenes*.

The addition of complement did not appear to result in any greater inhibition than was noted in its absence. Further tests carried out with a series of locally isolated strains of *Esch. coli* confirmed this observation, suggesting that an antibody-complement system effective against Gram-negative bacteria does not play an important role *in vitro*.

Table 5. *Effect of complement on the bactericidal activity of liver pus*

Organism	Inoculum: no. of organisms at zero time	Mixture	Bacterial count/ml. after incubation for 3 hr.	5 hr.	24 hr.
A. aerogenes	620	Pus + C′	10^3	3×10^4	6×10^8
		Pus + saline	10^3	2×10^4	$6{\cdot}5 \times 10^8$
A. aerogenes	62	Pus + C′	240	3×10^3	4×10^8
		Pus + saline	180	$2{\cdot}6 \times 10^3$	5×10^8
Esch. coli	960	Pus + C′	3×10^3	10^5	$4{\cdot}6 \times 10^8$
		Pus + saline	3×10^3	8×10^4	6×10^8
Esch. coli	96	Pus + C′	300	2×10^4	4×10^8
		Pus + saline	280	3×10^4	$3{\cdot}7 \times 10^8$

Complement control tubes had no bactericidal activity during the period of incubation. Initial inoculum—dilution of 12 hr. broth culture.

DISCUSSION

There are a number of possible ways whereby organisms may gain access to an hepatic abscess. These are: (*a*) by direct implantation from outside, e.g. by use of contaminated exploring needles, or, more rarely, by direct injury; (*b*) by direct spread from neighbouring viscera such as may occur following rupture of the abscess into bowel or lung. The former mode of rupture is uncommon with amoebic hepatic abscess but is often followed by a fatal termination; (*c*) by ascending spread from suppurative cholangitis; (*d*) by spread via the hepatic artery, and (*e*) by spread via the portal vein, from some area of infection in the drainage area or from ulceration of the bowel mucosa. In the presence of large bowel ulceration, transport of organisms to the liver would appear to be a likely and common occurrence. However, few reports are available concerning portal bacteraemia in ulcerative lesions of the gut. One of these is the study of Brooke (1959) on patients with ulcerative colitis. In a series of forty-four patients with this disease, fourteen had a positive portal blood culture and in two of these fourteen, concurrent liver biopsies yielded a growth of the same organism as was isolated from the blood. There is no mention of more than one culture having been performed in any patient in this series. However, it seems probable that portal bacteraemia under these circumstances may be a transient phenomenon and that repeated cultures might give a higher number of positive isolations. In an earlier report, Brooke & Slaney (1958) obtained specimens of portal blood at the commencement and at the end of operations involving handling of the gut. In nine patients with ulcerative colitis four yielded viable organisms in both specimens, whereas in a control series of fifteen patients without bowel lesions, one gave a growth of *Staph. saprophyticus* in both specimens, the remainder being sterile. Schatten, Desprez & Holden (1955), on the other hand, found eight positive portal blood cultures from twenty-five patients with intra-abdominal lesions, none of them involving the bowel, and they concluded that there is a continual seeding of the liver with bacteria derived from the gastro-intestinal tract.

If organisms can invade portal radicles from damaged bowel and be carried to the liver, the question arises as to why the incidence of bacterial infection in amoebic liver abscess should be so low since concomitant bowel ulceration is frequently found. The present results suggest that liver pus itself may not possess sufficient bactericidal activity to account for the low rate of infected abscesses. Amoebic liver pus in fact actively supports the growth of certain organisms *in vitro*, which are normal inhabitants of the gut. A number of other possible mechanisms may be considered which, either singly or in combination, may destroy organisms or prevent their being located in the abscess cavity.

It is possible that portal blood itself may have some bactericidal activity due to serum properdin and other naturally occurring factors. These mechanisms might be adequate to deal with a small infestation occurring infrequently, but are unlikely to be able to maintain sterility of portal blood entering the liver in the face of repeated and heavy contamination. Destruction of organisms may also occur following phagocytosis in Kupffer and other histiocytic cells as shown by Beeson, Brannon & Warren (1945). In this way organisms may be filtered off in areas of normal liver tissue adjacent to abscess sites. A certain amount of destruction of organisms may also take place in the abscess cavity itself. Microscopic examination of amoebic hepatic pus shows considerable numbers of polymorphonuclear and mononuclear cells, presumably the result of a reaction to the presence of dead parenchymal cells. If this mechanism does play a part it would seem that there must be additional factors involved which do not operate *in vitro*. A further possible explanation is that organisms fail to enter the abscess cavity even though they may remain viable in the surrounding normal liver tissue. Histological examination of the walls of amoebic hepatic abscesses usually reveals a ragged necrotic lining surrounded by a zone of dead but intact liver cells which have not undergone liquefaction. This zone, which in some instances may be several millimetres in thickness, is devoid of blood, the vessels having undergone thrombosis. In addition, the necrosis of liver cells leads to a collapse of the supporting reticulin framework often with the appearance of a zone of compact reticulin in the abscess wall. It is suggested that this avascular region presents an adequate barrier to the passage of organisms into the abscess cavity, and that this represents one of the main reasons for the usual bacteriological sterility of the pus from these abscesses.

The presence of organisms in liver pus on second or subsequent aspiration, where the initial specimen was sterile, has been explained by Maddison *et al.* (1959) as resulting from infection from without, along the needle track. Doubtless this does happen but a second explanation, based on the last hypothesis considered above, occurs to us. It is suggested that aspiration is followed by a release of tension in the abscess resulting in a breakdown of the surrounding avascular zone of necrotic liver tissue, with the result that blood, containing bacteria, is allowed into the cavity. This sequence of events in fact is almost certainly the explanation for the appearance of so-called 'anchovy sauce' pus. Abscesses opened at autopsy do not usually present this appearance. Roach (1958), for example, found that more than 90 % of amoebic abscesses at autopsy were yellow in colour. Further support for this suggestion is found in the observation that live amoebae, which are most

plentiful in the ragged lining zone, are more often found in the terminal portions of aspirated material than in the earlier fractions, suggesting that collapse of the lining zone has shed them into the cavity. Consequently, it is possible to envisage that organisms in the surrounding liver tissue or portal radicles may reach the abscess cavity following disruption of this barrier zone. In this event secondary bacterial infection is often endogenous rather than exogenous in origin, as is suggested by the high incidence of infection with members of the family Enterobacteriaceae detailed in Table 1.

Sh. sonnei, *Sh. boydii*, *Staph. pyogenes* and *Strep. faecalis* attained to stabilized population densities short of the maximum populations found in control broth cultures. This phenomenon was not observed with *Esch. coli*, *Salm. typhimurium* or *A. aerogenes*. Such inhibition may represent a limiting nutrient factor in liver pus for certain strains, some form of toxic inhibition or possibly some type of adaptive response on the part of the organism itself to the environment, similar to that suggested by McDermott (1958) in relation to the phenomenon of microbial persistence. Further information on the sizes of microbial populations in amoebic liver abscesses *in vivo* is required in the elucidation of this speculation.

SUMMARY

1. Bacterial infection of amoebic hepatic abscess is a relatively uncommon event.

2. Investigations to demonstrate a possible bactericidal effect of liver pus *in vitro* indicated that small inocula, especially of young cultures, of *Sh. sonnei*, *Staph. pyogenes* and an organism of pseudomonas species were rendered sterile. Organisms such as *Esch. coli*, *Salm. typhi*, *Salm. typhimurium*, *Sh. boydii* and *Strep. faecalis* showed a drop in viable count during the first few hours of incubation followed by a logarithmic increase. The strain of *A. aerogenes*, on the other hand, was not decreased in numbers during the first few hours of incubation and showed a prolonged lag phase followed by a logarithmic increase in numbers. *Shigella sonnei*, *Staph. pyogenes*, *Sh. boydii* and *Strep. faecalis* attained to maximum population densities which were less than those obtained in control broth tubes and less than those of other organisms in liver pus.

3. It is suggested that secondary bacterial infection of amoebic liver abscess is often endogenous in origin, occurring after primary aspiration due to disintegration of the lining wall of the cavity, allowing the entrance of portal blood containing organisms from the damaged bowel.

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