EXPERIMENTAL STUDIES ON THE IMMUNE RESPONSE TO EHRLICH'S ASCITES CARCINOMA

by

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With due permission of the senate of the University of Bergen to be publicly discussed on 13th May 1964 for the degree of *Doctor medicinae*

BERGEN 1964

ACKNOWLEDGMENTS

My thanks are due mainly to Professor E. Waaler who made it possible for me to carry out this work while a research assistant in his department. His encouragement, criticism and common sense have been invaluable. I am also very grateful to Dr. Glück for his advice and never failing enthusiasm.

I am much obliged to Professor L. Kreyberg of the University of Oslo, who kindly supplied the forebears of the mice used in the experiments, and to Professor C. Ahlström of the University of Lund, Sweden, who kindly provided a transplant of the tumour. I would also like to thank Professor P. Armitage of the London School of Hygiene and Tropical Medicine and Dosent Humerfelt of this University for statistical advice.

I am indebted to the Bergen University Library for supplying the literature I have needed, to Miss R. Sandtorv for help with the protein estimations, to Mr. A. Andersen for making up solutions, to Mr. T. Christensen for photographic help and to Mr. E. Hornenes who looked after the mice, and would like to take this opportunity of thanking them.

If my husband and children have suffered from the effects of having a "working" wife and mother I hope they will forgive me, and allow me to dedicate this work to them. Lastly, my parents deserve my whole-hearted thanks for letting me study medicine and come to Norway.

14. 3. 1968.

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INTRODUCTION

In 1954 Burnet (5) put forward the idea that in some cases the body might be able to react against its own tissues. In this connection he did not mention the possibility of such an immune response to tumour tissue, but on reading it the present author was forcibly struck by the idea that if such a reaction was thought to occur against normal tissues it might also be possible to produce a similar reaction against cancer cells.

Although such a response to normal tissue could be obtained by the repeated injection of normal tissue, its production was greatly facilitated by the addition of adjuvants, in particular Freund's adjuvant (14), to the tissue injected (33,45). In 1955 Witebsky reported the first attempt to induce immunity to human cancer by this means (61). His experiments were followed up by Graham and Graham (18,19). As these trials were on cancer patients they were of necessity uncontrolled. In the same year Fink, Smith and Rothlauf (11) reported similar experiments using a chemically induced transplantable mouse sarcoma. The present work was therefore started in an attempt to provide further animal experiments in this field.

Mice were chosen as the experimental animal and Ehrlich's ascites carcinoma as the cancer. The tumour was considered suitable, firstly, as it could be expected to grow in all mice (34), and secondly, as the survival time of the animals after intraperitoneal injection of the tumour was known to be determined by the tumour cell dose (37). Thus if the cell dose were held constant an immune response might be reflected in the growth of the intraperitoneal tumour, and hence in the survival time of the mice. The results of preliminary experiments showed, however, that the resistance of the mice to the tumour, as judged from their survival time, far from being increased by the treatment, was decreased. On the other hand, some of the control experiments brought to light findings that it was thought might add to our knowledge of the immune response to Ehrlich's ascites carcinoma.

The present work consists of a collection of nine papers that grew out of these findings. Each paper deals with a problem that arose in, or was closely associated with, the previous experiment. The problems are all related to the vexed question of immunity to tumour growth.

This question first arose at the turn of the 19th. century in connection with the growth of the newly discovered transplantable tumours (47, 44, 29, 30, 31, 32, 8). These tumours provided material, that had hitherto been lacking, that opened up the possibility of carrying out animal experiments on the growth of tumours. Morau (44) was the first to manage to transfer such a tumour from mouse to mouse for many transplant generations. Then came Jensen with his transplantable mouse carcinoma. Others, including Ehrlich and Apolant, Bashford, Murray, Flexner, Jobling and Borrel soon followed with their work on tumour transplantation (see 62).

In addition there was a Norwegian pioneer in this field and it is only natural that his work should be quoted here, in particular as this man was Magnus

Haaland who was later head of the Institute from which the present work now comes. These are by no means the only reasons for quoting his views on tumour immunity. His work has since shown him to have been in many ways far ahead of his time in his understanding of this problem which interested him deeply at the beginning of his career.

Haaland was introduced to the field of tumour transplantation by Borrel in 1903 and at the same time introduced to the study of immunology by Metchinoff (21). In the course of the next few years he also studied under Aschoff, Behring, Ehrlich and Neisser before he went to London to continue his work under Bashford and Murray. At this time bacteriology was a well established science while immunology was still in its infancy. Thus the pioneers in the field of tumour transplantation were bacteriologists, and in many cases, for example Pfeiffer's and Ehrlich's and in particular Haaland's, their interest in tumour transplantation vied with their interest in bacteriology and immunology.

Morau's tumour, Jensen's tumour and later Ehrlich's tumours were easily propagated and, as they were mouse tumours, it is natural that much of the early work on tumour transplantation was confined to mice — though other transplantable tumors did exist (62). It soon became clear that the transplantable mouse tumours would not grow in all mice (9). Some thought that this might be due to the method of transplantation, others to the age of the mice or the virulence of the tumour (62), and some, including Haaland, thought it might be due to the genetic relationship of the host to the transplant (22).

Haaland's experiments on pigeon molluscum (21) introduced him to the concept of a virus tumour and to the finding that once such a tumour had grown and regressed the host was immune to a further injection of the same tumour. The finding that transplantation immunity of similar type occurred with mouse tumours, and that it was more or less specific to the tumour used, helped to convince him that a common tumour virus did not exist (23). Bashford's experiments on inducing immunity to tumours by the previous injection of normal tissue (4) strengthened his conviction.

By this time it was clear that the explanation of the spontaneous absorption of a tumour transplant lay in the host's resistance and not in the tumour itself (23). The next step was to induce host resistance to a tumour that usually grew progressively. Here Ehrlich, on the basis of the analogy of inducing bacterial immunity by the previous injection of attenuated organisms, used an avirulent form of a tumour to produce immunity to a subsequent virulent transplant (9). Haaland followed this up by using damaged tumour cells (24). But he found that dead cells — far from inducing immunity — enhanced tumour growth and his findings were supported by the results of other workers (55). The idea thus arose that immunity to the transplantation of tumour tissue or of normal tissue was extremely fragile, very easily destroyed, and hence bound to the living cell. It was later found that some forms of cell damage are compatible with the production of immunity (54). It was realized that this immunity differed from bacterial immunity as no evidence of antitumour antibody was to be found in the serum of immune animals (63,25). The growth of metastases to spontaneous tumours prompted Haaland to state that these tumours could not be different enough from the host to enduce it to react against them (23).

Little's postulation of the genetic theory of transplantation, which he and Snell (53) were able to verify, has clarified many of the previous findings concerning transplantation immunity. It is now accepted that a transplant that

is absorbed, be it tumour or normal tissue, must differ genetically from its host. The protein produced by the gene that is different will be recognized as "non-self" (5) by the host, which will then set its defences in swing to render the foreign protein innocuous. As this protein is contained in the cells of the transplant this process will lead to the disruption of cell function: the host defences will have succeeded in bringing about the rejection of the transplant. The result of transplantation will depend mainly on the number of proteins in the transplant that are regarded as foreign by the host. Thus if a tissue is considered as made up of species specific, organ specific and self specific proteins a transplant between highly inbred animals will in all probability be successful as all three component protein types should be identical, a transplant between non-related animals of the same species (a homograft) would contain different self specific proteins, so rejection would be expected; while in a transplant between animals of different species so many proteins would be recognized that rejection would be the rule.

As Haaland (23) pointed out, and as Barrett (3) found it necessary to stress once again in 1956, immunity to a transplantable tumour is transplantation immunity and cannot be considered to be due to any cancer specific antigen in the transplant. The idea of tumour immunity, i.e. immunity of a host to its own spontaneous tumour, as distinct from transplantation immunity, implies tumour rejection. This hypothesis is based on the idea that even autologous tumours, by virtue of their very existence, must be different from their hosts (64). The genetic constitution of an autologous tumour could then be expressed as being made up of species specific, organ specific, self specific and tumour specific protein; the latter having come about as the result of a gene mutation, chemical damage, or the entry of a virus into the cell, producing the same end result — alteration in a gene. The host would then be expected to react against the altered protein produced by this gene. This protein would be "changed-self", and as such recognized as non-self. The majority of experiments to determine the antigenic composition of tumour cells have shown that they possess fewer antigens than normal cells (15, 16, 25, 46). On the other hand evidence is collecting that tumour cells, in addition, also contain antigens that normal cells lack (64, 65, 12, 28), virus tumours being an extreme example of such a situation.

However, though some spontaneous tumours have now been shown to contain antigens lacking in the normal tissues of the host, there is still little evidence that these antigens give rise to antibody, as they theoretically should. (It has been suggested that cancer patients may lack the ability to respond to the antigenic stimulus provided by the tumour (17). This is, however, akin to denying the recognition of "changed-self" as non-self that forms the basis of the postulation of tumour immunity). On the other hand on considering clinical evidence of tumour immunity there is more to be found in its support. Firstly there are the few reports of spontaneous remission of histologically proven cancers (58). Then there are the reports of the remission of cancer following infectious disease (10). These are spectacular findings and many doubt their veracity. But no one can doubt the well known fact that tumours that appear identical histologically behave very differently clinically — indicating that some patients have more resistance to tumour growth than others. In addition the number of tumour cells found in the blood stream appears to be greatly in excess of the number of metastases they produce (49), and seeding at operation is less frequent than might be expected (51). See also reviews (56, 43, 57, 85).

If tumour immunity is to be postulated at all it is unreasonable to expect that the tumour specific antigen would not be recognized by the host. Is it not more likely that such a reaction takes place but is abortive? In other words is it not possible that we are dealing with a further example of immunological tolerance — similar to the types that Medawar (42) has classified as "states in which there is some reason to believe that immunological activity has been thwarted"?

This idea that the host may produce specific antibody, but that the immune reaction does not result in cell lysis, opens up many possibilities for experimentation and there is already evidence in its favour. Firstly, it has been shown that antibody can become fixed to tumour cells in the absence of complement (these cells then show agglutination) — and that the addition of complement is needed for cell damage (lysis) to result (13, 7, 20, 40). The situation of the tumour cells in such a case is analogous to that of amboceptor treated erythrocytes. Secondly, it has been shown that the serum of some cancer patients lacks factors present in normal serum; one of these factors is thought to be one of the components of complement (39). Further it has been shown that tumour cells in the presence of specific antibody, both in vivo and in vitro, are not damaged in the absence of sufficient complement (60,2). The idea that tumour antibody is produced but that its action is abortive could explain both the progressive growth of and the lack of demonstrable serum antibody to spontaneous tumours; the search for the latter being, in effect, analogous to a search for antibody in the supernatant fluid from an adsorption experiment. In addition states of immunological tolerance are thought to continue only as long as the antigen persists in the body (42). This could explain the immune response of animals to a second graft following the removal of a previous tumour graft.

A transplantable tumour that grows progressively behaves clinically in the same way as a spontaneous tumour. It does not follow the basic law of transplantation immunity, that a homograft, i.e. a transplant between unrelated animals of the same species (52), will regress. This has been put down to the fact that such tumours, during the process of repeated transplantation and adaptation to life in hostile surroundings, have lost so many antigens that only those that are species specific remain (27). Thus the tumours pass unrecognized on transplantation — being accepted in the same manner as an autograft, or, for that matter, a spontaneous tumour.

Ehrlich's ascites carcinoma is a tumour of this type. It originated as a spontaneous mammary carcinoma, and is said to have arisen at some time between 1903 and 1905 at the Frankfurt Institute. It was then known as Ehrlich's solid mouse carcinoma. It arose in a heterozygous mouse and was transplanted serially, subcutaneously, in market mice. All records of its transplantation were lost in World War II (50). However, in 1936 Fischer, in Copenhagen, had received a transplant from Frankfurt and kept it in vitro for 14 years with only occasional mouse passages. In 1948 Klein (36, 38), in Stockholm, following the example of Loewenthal and Jahn in 1932 (41), converted the tumour he obtained from Fischer into the ascitic form (26). Klein later sent a transplant to Ahlström (1) in Lund, from whom the present author received a transplant in 1959 — and started the attempts to induce immunity to this tumour that led to the findings reported in the present work.

The statistical methods used throughout are those common to standard textbooks.

The experimental animals were selected in the following way. All the available adult animals under 6 months old were weighed and placed in weight groups (to the nearest gram) for each sex. They were then selected at random from these weight groups, in descending order of weight. If more than one experimental group was needed mice of the same weight and sex received the same number in each group. In this way the mean weight and its standard deviation were the same in all groups.

The colony of mice was kept closed. Their housing conditions were constant throughout the time the experiments were in progress. At the time of the first experiment in this series they were fed on bread, crushed oats and a mixture of equal parts of fresh milk and water. They were also given raw swede on Saturdays. Between the first and second experiments the diet was changed, for convenience, to pellet form (Kambo. Forblanding I.). The supplement of raw swede was continued and they were given water to drink. The mice thrived and intercurrent infection was not troublesome.

Reprinted from
The British Journal of Cancer,
1961, Vol. XV, p. 336

THE SURVIVAL TIME OF MICE WITH EHRLICH'S ASCITES CARCINOMA RELATED TO THE SEX AND WEIGHT OF THE MOUSE, AND THE BLOOD CONTENT OF THE TUMOUR

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Received for publication April 20, 1961

It appears to be generally accepted that Ehrlich's ascites carcinoma will grow in all mice (Karnofsky, 1953), with no spontaneous remissions (Lettré, 1941). Klein and Révész (1953) showed that a minimum of 400,000 tumour cells was necessary to obtain ascitic growth in all cases. However, although the death of the mouse is certain within a time limit dependent on the tumour cell dose, the scatter in the survival time has not been investigated.

The tumour grows equally well in male and female mice, although Ahlström and Ising (1955) have shown that there is a difference in its growth in male and female hamsters. The tumour cells and the inflammatory cells present in the tumour ascites have been investigated at the expected median survival time of the mice (Klein, 1950), but no reference has been found to any investigation into the amount of blood present in this ascites, or its significance.

This experiment was therefore designed to see if the sex and weight of the young adult mice kept at this Institute had any bearing on their survival time after the intraperitoneal injection of Ehrlich's ascites carcinoma, and further to see if there were any difference in the amount of blood in the tumours of mice dying spontaneously at different times after injection.

MATERIAL AND METHODS

Twenty-five male and 25 female mice were used. All were adult but under 6 months old. They were taken from a closed colony of previously inbred white mice obtained from Professor Kreyberg in Oslo.

The Ehrlich's ascites carcinoma was originally obtained from Professor Ahlström in Lund who had earlier got it from Klein in Stockholm. At the time of the experiment the tumour was in its 67th transplant generation here.

All the mice were weighed and marked. Each mouse was then given one intraperitoneal injection of 0·1 ml. tumour ascites (a tumour cell dose of 1,860,000/mm.c.) taken from one mouse. This mouse had been injected with Ehrlich's ascites carcinoma 10 days before.

The mice were kept in cages of 5. When a mouse died the survival time was recorded in days. Then the abdomen was opened and the tumour ascites removed and measured. One ml. of the fluid was centrifuged at 2,400 r.p.m. for 45 minutes. The volume of the red blood cells that formed a dark layer at the bottom of the Wintrobe tube was taken as an estimate of the amount of blood present per ml., and recorded as a percentage of the total volume in the tube.

RESULTS

Fig. 1 shows the distribution of deaths related to time. It will be noticed that this curve has two peaks, marked a and b. If the mean survival time of the total series, 10·5 days, is taken as the dividing point, the mean survival time is 7 days for group a and 13·03 days for group b.

Table I shows the mean survival time and its standard deviation (SD) for the mice (a) dying before the mean survival time for the total series (corresponding to the first peak in Fig. 1) compared to that of the mice (*b*) dying after this mean survival time (corresponding to the second peak). The table also gives the mean

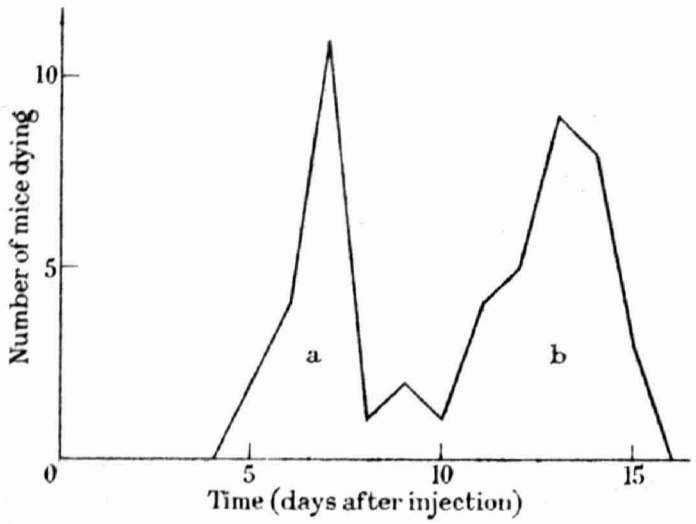

FIG. 1.—The distribution of the deaths of 50 mice dying after the intraperitoneal injection of Ehrlich's ascites carcinoma.
a. Peak corresponding to group a.
b. Peak corresponding to group b.

amount of blood/ml. tumour ascites and the final volume of the tumour ascites, with the SD of these means for groups a and b. The standard error (SE) of the actual difference between the means of the groups, the t and P values are shown for all three factors. These findings indicate that the difference between both

TABLE I.—*The Survival Time of the Mice in Groups a and b, and the Blood Content and Final Volume of the Tumours, Showing the Number of Mice, the Mean Values with SD, the SE of the Difference Between the Group Means, the* t *and* P *Values.*

x	Group	n	$\bar{x}$	$SD_{\bar{x}}$	$SE_{\bar{x}_a-\bar{x}_b}$	t	P
Survival time (days)	a	21	7·0	1·196	0·346	17·42	0·001>P
	b	29	13·03	1·225			
Blood vol./ml. ascites (%)	a	21	12·23	4·096	1·125	9·14	0·001>P
	b	29	1·91	1·114			
Volume of ascites (ml.)	a	21	2·74	1·146	1·770	2·564	0·02>P>0·01
	b	29	7·28	2·983			

the survival time and the amount of blood present in the tumour in both groups is very highly significant statistically, while the difference in the final volume of the tumour ascites is also significant.

Fig. 2 shows the regression lines for the scatter diagrams when (1) the volume of tumour ascites is related to the survival time ($y = 51{\cdot}68 - 2{\cdot}869x$), and (2) the blood volume/ml. tumour ascites is related to the survival time ($y = 22{\cdot}17 - 1{\cdot}54x$). From this figure it is evident that the amount of tumour present in the dead mice is greater in those surviving longer, while the amount of blood in the tumour is greater in the mice dying after a short survival time.

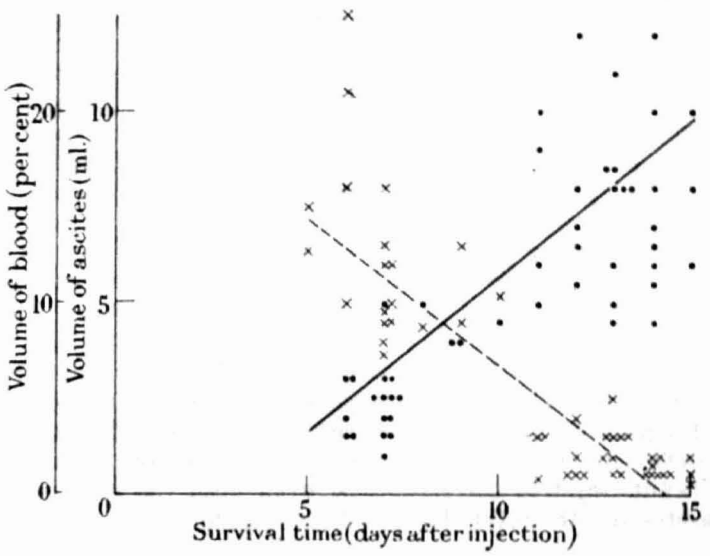

FIG. 2.—Scatter diagram and regression line for :—
1. Volume of tumour ascites (●) related to survival time (———).
2. Blood volume/ml. ascites (×) related to survival time (– – – –).

Table II gives the mean values for the starting weight, survival time, volume of tumour ascites, blood volume/ml. ascites and total blood volume in the ascites for the total series (♂ + ♀) and for the males and females alone. In addition to the number of animals involved, the mean values with SD, the SE of the differences between the male and female means, the t and P values are shown.

From this table it is evident that although the difference in the mean starting weight for the male and female series is highly significant statistically, the differences in the mean survival time, volume of tumour ascites, volume of blood/ml. tumour ascites and total blood volume in the tumour ascites are not significant.

Table III shows the correlation between the starting weight and the final volume of tumour ascites, and between the survival time and the following factors : the starting weight, the volume of tumour ascites, the volume of blood/ml. ascites and the total blood volume in the ascites. The table gives the number of animals used, the correlation coefficient (r), the t and P values for the various factors.

It can be seen from Table III that the correlation between the starting weight and the final volume of tumour ascites is not statistically significant in the male

TABLE II.—*The Distribution of the Factor* x *for the Total, the Male and the Female Series, Giving the Number of Mice, the Mean Values with SD, the SE of the Difference between the Male and Female means, the* t *and* P *Values.*

x	Series	n	$\bar{x}$	$SD_{\bar{x}}$	$SE_{\bar{x}_♂-\bar{x}_♀}$	t	P
Starting weight (g.)	♂+♀	50	18·58	4·858	1·374	2·833	0·01>P>0·001
	♂	25	20·52	1·691			
	♀	25	16·64	6·075			
Survival times (days)	♂+♀	50	10·5	3·208	0·9203	1·087	0·1>P>0·05
	♂	25	11·0	3·349			
	♀	25	10·0	3·222			
Volume of ascites (ml.)	♂+♀	50	5·57	2·292	0·8258	1·380	0·2>P>0·1
	♂	25	6·14	3·211			
	♀	25	5·0	2·466			
Blood/ml. ascites (%)	♂+♀	50	6·0	5·771	1·635	0·1761	0·9>P>0·8
	♂	25	5·98	6·059			
	♀	25	6·02	6·020			
Total blood (ml. × %)	♂+♀	50	21·54	13·07	3·696	0·5195	0·7>P>0·6
	♂	25	21·17	12·71			
	♀	25	23·09	11·25			

series. However, for the female and the total series this correlation is significant at the 5 per cent level. The correlation between the survival time and the starting weight is not significant in any of the series. There is a highly significant positive correlation between the survival time and the volume of tumour ascites, and a highly significant negative correlation between both the survival time and the blood/ml. ascites and the total blood in the ascites for all the series.

TABLE III.—*Correlation Between Factors* x *and* y, *Showing the Number of Animals Used, the Correlation Coefficient* (r), *the* t *and* P *Values, for the Total, the Male and the Female Series.*

x	y	Series	n	r	t	P
Starting weight (g.)	Volume of ascites (ml.)	♂+♀	50	0·3226	2·258	0·05>P>0·02
		♂	25	−0·1733	0·8492	0·4>P>0·3
		♀	25	0·5077	2·488	0·05>P>0·02
Survival time (days)	Starting weight (g.)	♂+♀	50	0·07679	0·537	0·6>P>0·5
		♂	25	−0·1547	0·7582	0·5>P>0·4
		♀	25	0·2134	1·046	0·4>P>0·3
Survival time (days)	Volume of ascites (ml.)	♂+♀	50	0·7998	5·593	0·001>P
		♂	25	0·7632	3·74	
		♀	25	0·8472	4·152	
Survival time (days)	Blood/ml. ascites (%)	♂+♀	50	−0·8506	5·94	0·001>P
		♂	25	−0·9166	4·49	
		♀	25	−0·7539	3·694	0·01>P>0·001
Survival time (days)	Total blood (% × ml.)	♂+♀	50	−0·6992	4·89	0·001>P
		♂	25	−0·7338	2·596	0·01>P>0·001
		♀	25	−0·6750	3·308	

DISCUSSION

Although the mice used in this experiment were all taken from the same closed colony and each received the same dose of Ehrlich's ascites carcinoma cells from the same mouse, some scatter in their survival time was expected. The two-peaked curve that was obtained when the number of deaths was related to the time after injection suggests, however, that the mice dying of this tumour fall into two distinct groups, as is evident from Fig. 1. The SD in both groups is low and the means are separated by as much as 6·03 days. The chance that both these groups were taken from the same population is less than 1 : 1000. Thus the mice used here react in two ways to the same treatment.

This experiment shows that neither the sex nor the weight of the mouse influenced the survival time. There was, however, a highly significant positive correlation between the survival time and the final volume of the tumour, showing that the tumour grows progressively. This is in accordance with Klein's (1950) findings. There was also a positive correlation between the final volume of tumour and the starting weight of the mouse. This correlation is significant at the 5 per cent level in the total and female series, but not in the male series. This is probably accounted for by the greater scatter in the weights of the female mice and by the fact that the female mice were, on the whole, smaller than the male. This shows that the smaller mice produced smaller amounts of tumour.

When the ascitic variant of the Ehrlich mouse carcinoma was first obtained the formation of " a huge ascites of milky or bloody character " was described occurring 10–14 days after intraperitoneal inoculation (Loewenthal and Jahn, 1932). Klein (1950) quotes this statement but makes no further reference to the blood content of the tumour. In his later work on the growth curves of ascites tumours (Klein and Révész, 1953) he states that there was " often a slight admixture of erythrocytes " in the Ehrlich ascites carcinoma, but this was not studied further and in some experiments blood stained fluids were not investigated. Kun, Talalay and Williams-Ashman (1951) say, with regard to this tumour, " Characteristically, the fluid is milky white, has a tendency to clot, and occasionally is grossly hemorrhagic. Fluids containing more than about 15 per cent erythrocytes of the total cells were discarded ".

The present experiment shows that the amount of blood is related to the survival time, a highly significant negative correlation being present between the blood volume/ml. ascites and the survival time. It may be argued that the blood volume might remain constant while the tumour volume increased, giving in itself a negative correlation. But if the percentage blood/ml. is multiplied by the volume of tumour ascites present, and this figure (the total blood present) is correlated to the survival time, the negative correlation is still highly significant. Referring this finding back to the two groups discussed previously we find that the blood content of the tumours in groups a and b differs to a very highly significant extent, the tumours of the mice with a short survival time containing more blood than those of mice surviving longer.

Apitz (1934) tested the idea that haemorrhage into the solid Ehrlich carcinoma might be due to anaphylaxis but obtained negative results on a small series of stock mice. From the results of the present experiment it is possible to say that the blood content of the Ehrlich ascites carcinoma is related to the survival time

and is an expression of the finding that the mice react to the tumour in two ways but it is not possible to say why they do so. Further experiments on this question are in progress.

SUMMARY

Twenty-five male and 25 female mice were injected intraperitoneally with the same cell dose of Ehrlich's ascites carcinoma. This was taken from one mouse. It was found that the mice fell into two distinct groups as regards survival time, although the survival time was not influenced by the sex or weight of the mouse. The amount of tumour was less in the smaller mice. A positive correlation was found between the survival time and the final tumour volume, and a negative correlation between the survival time and the volume of blood in the tumour.

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Made and printed in Great Britain for
H. K. Lewis & Co. Ltd., by
Adlard & Son, Ltd., Bartholomew Press, Dorking.

Reprinted from
The British Journal of Cancer,
1961, Vol. XV, p. 665

THE SIGNIFICANCE OF THE BLOOD CONTENT OF THE EHRLICH ASCITES CARCINOMA

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Received for publication June 19, 1961

It has been shown previously (Hartveit, 1961) that the adult mice used at this Institute show either a short survival time and a very haemorrhagic tumour or a long survival time and a relatively asanguinous tumour, following the intraperitoneal injection of Ehrlich's ascites carcinoma. This negative correlation between the survival time and the blood content of the tumour is not dependent on the sex or weight of the mouse. As this tumour is said to grow progressively in almost all strains of mice (Karnofsky, 1953) it was decided to investigate the cause of this difference in survival time and blood content found in our mice.

In 1934 Apitz tested the theory that haemorrhage into the solid Ehrlich carcinoma might be due to anaphylaxis, but he failed to produce haemorrhage by this means. He also failed to produce haemorrhage as the result of treatment with histamine. Later Barrett (1942) investigated the relationship between anaphylaxis and sarcoma 37. He was able to show that the anaphylactic shock produced in previously sensitized mice in response to horse serum was accompanied by haemorrhage into actively growing transplants of this tumour. The reaction did not occur in non-sensitized animals. He also produced haemorrhage into the tumour in strain A mice in response to intraperitoneal histamine. The haemorrhagic reaction was not confined to the tumour but was also present, to a lesser extent, in the stomach and small intestine. Barrett's experiment suggests that haemorrhage into a tumour may be the result of the combination of a foreign antigen with antibody present in the tumour.

The mice used in this Institute are heterozygous and also of different genetic make-up from Ehrlich's ascites carcinoma. It was thought that their differences in reaction to the tumour might represent different degrees of genetic difference, and thus different degrees of resistance to the homotransplant.

Ludford (1931) and Andervont (1936) have shown that resistance to tumour transplants can be abrogated by vital staining with trypan blue. More recently many authors (reviewed by Toolan, 1953) have shown that it is possible for homotransplants to grow in animals treated with cortisone. The following experiment was planned in an attempt to reduce the resistance of mice to a tumour homotransplant (i.e. intraperitoneal Ehrlich's ascites carcinoma) by both the methods mentioned above, to see if this treatment would influence the survival time of the mice and the blood content of the tumour ascites.

MATERIAL AND METHODS

The mice used were taken from a closed colony of previously inbred white mice. All were adult, under 6 months old. The Ehrlich ascites carcinoma was originally

obtained from Professor Ahlström in Lund, and, at the time of the experiment, was in its 70th transplant generation here.

Three groups of mice (I, II and III), each containing 15 males and 15 females, were numbered 1–15 for each sex in each group ; mice of the same sex and weight (to the nearest gram) in each group receiving the same number. In this way the mean starting weight of the animals in all 3 groups was the same, being 20·8 g. (S.D. 1·237).

Each of the mice was given one intraperitoneal injection of 0·1 ml. of Ehrlich's ascites carcinoma taken from a male mouse of the 70th transplant generation. This mouse, which had received the tumour 10 days before, had 9·5 ml. of tumour ascites which was slightly blood stained, with a tumour cell count of 2,050,000/cu. mm. The cells all appeared viable, i.e. they did not take up eosin from a 1 : 2,000 solution of eosin in Tyrode's solution (Schrek, 1936) : they did not clump and showed no abnormalities in films stained with haematoxalin and eosin. When the injections were complete, about one hour after the donor mouse had been killed, the tumour cells in the remaining fluid were all still viable.

Some of the mice were also given subcutaneous treatment as follows :—

Group I—the control group—received no subcutaneous treatment.

Group II—the vitally stained group—were each given 0·5 ml. of a 0·5 per cent solution of trypan blue in sterile distilled water, subcutaneously on the back. The first injection of trypan blue was given 7 days before the mice were injected with tumour ascites. The trypan blue injections were repeated 2 and 4 days later, and thereafter weekly until all the mice in the group were dead.

Group III—the cortisone treated group—received a suspension of cortisone acetate as a subcutaneous injection on the back. The dose was equivalent to 25 mg./kg. starting weight. The first injection was given 6 days before the mice were injected with tumour ascites. The cortisone injections were repeated daily until all the mice in the group were dead.

Three additional control groups were set up (1, 2 and 3). Each of these groups consisted of 5 male and 5 female mice of similar age and weight to those in the three main groups. Each of these groups was given the same subcutaneous treatment as the corresponding Roman figure group (i.e. none, trypan blue, and cortisone, respectively), but none of the mice were injected with tumour ascites.

The survival time of each mouse that died in every group was recorded. When the mice in the Roman figure groups died the tumour ascites was removed and 1 ml. of this centrifuged at 2,400 r.p.m. for 45 minutes. The volume of the red blood cells at the bottom of the Wintrobe tube was taken as an estimate of the amount of blood present/ml. and recorded as a percentage of the total volume in the tube.*

RESULTS

Table I shows the sex difference in the survival time and blood content of the tumour ascites in the mice in groups I, II and III, all of which died. The table gives the values for the total, male and female series, with the number of mice, the mean value with S.D., the S.E. of the actual difference between the male and female means, and the t and P values for this difference.

* One mouse in group II died of trauma the day after the injection of the tumour and was excluded from subsequent calculations.

TABLE I.—*The Sex Differences in Survival Time and Blood Content of the Tumour Ascites in the Three Groups*

x	Group	Series	n	$\bar{x}$	S.D.$\bar{x}$	S.E. difference $\bar{x}_{\sigma}$ & $\bar{x}_{\varphi}$	t	P
Survival time (days)	I	♂+♀	30	11·03	2·683			
		♂	15	10·8	2·186	1·013	0·4862	0·7>P>0·6
		♀	15	11·3	3·253			
	II	♂+♀	29	12·24	3·332			
		♂	14	12·9	2·901	1·33	0·9773	0·4>P>0·3
		♀	15	11·6	4·188			
	III	♂+♀	30	14·93	4·528			
		♂	15	11·8	3·571	1·233	5·077	0·001>P
		♀	15	18·06	3·17			
Blood content of ascites (vol. %)	I	♂+♀	30	4·3	4·638			
		♂	15	4·4	5·144	1·736	0·0806	P>0·9
		♀	15	4·26	4·331			
	II	♂+♀	29	1·717	4·286			
		♂	14	2·036	3·782	1·702	0·2974	0·8>P>0·7
		♀	15	1·53	5·304			
	III	♂+♀	30	1·216	2·616			
		♂	15	0·9	2·569	0·9806	0·6424	0·6>P>0·5
		♀	15	1·53	2·798			

The number of mice in each group and series is shown, the mean value for the factor under consideration (x) with its S.D., the S.E. of the actual difference between male and female means, and the t and P values for this difference.

The mean survival time for the total series was shortest in the control group I (11·03 days, S.D. 2·683), and longest in the cortisone treated group III (14·93 days, S.D. 4·528). The vitally stained group II fell between these two extremes (12·24 days, S.D. 3·332). The sex difference within the groups was not statistically significant in groups I and II (0·5 and 1·3 days respectively), while it was highly significant in group III (6·26 days, 0·001 > P). The mean blood content of the tumour ascites was highest in the control group I (4·3 per cent, S.D. 4·638), and lowest in the cortisone treated group III (1·216 per cent, S.D. 2·616). Once again the vitally stained group II showed an intermediate value (1·717 per cent, S.D. 4·286). The sex differences were not significant.

TABLE II*a*.—*Comparison of the Differences in Survival Time Between the Three Groups*

	Comparison between groups x and y		Series	n_x	n_y	Difference between $\bar{x}$ and $\bar{y}$	S.E. difference between $\bar{x}$ and $\bar{y}$	t	P
Survival time (days)	I	II	♂+♀	30	29	1·21	0·7891	0·1533	0·9>P>0·8
	I	III	♂	15	15	1·0	4·187	0·2388	0·9>P>0·8
	I	III	♀	15	15	6·76	1·172	5·768	0·001>P
	II	III	♂	14	15	1·1	1·204	0·9137	0·4>P>0·3
	II	III	♀	15	15	6·46	1·356	4·764	0·001>P

The difference in survival time between the groups for the total series, or for the male and female series separately when their difference is significant, is shown, with the number of mice used and the S.E., t and P values for the difference.

Table II*a* compares the differences in survival time between the three main groups, giving the difference between the groups for the total series, or for the male

and female series separately when their difference is significant, with the number of mice used, the S.E., t and P values for the difference.

The difference in survival time of the mice in groups I and II, total series (1·21 days) is not statistically significant. In groups I and III this difference (1 day) is not significant for the male mice, but the difference for the female mice in these groups (6·67 days) is highly significant ($0{\cdot}001 > P$). The difference between groups II and III for the male mice (1·1 days) is not significant, but that for the females (6·46 days) is highly so ($0{\cdot}001 > P$).

TABLE IIb.—*Comparison of the Differences in Blood Content of the Tumour Ascites Between the Three Groups*

	Comparison between groups x and y		Series	n_x	n_y	Difference between $\bar{x}$ and $\bar{y}$	S.E. difference between $\bar{x}$ and $\bar{y}$	t	P
Blood content of ascites (vol. %)	I	II	♂+♀	30	29	2·583	1·161	2·224	0·05>P>0·02
	I	III	♂+♀	30	30	3·184	0·9705	3·281	0·01>P>0·001
	II	III	♂+♀	29	30	0·501	0·7416	0·675	0·5>P>0·4

The difference in blood content between the groups is shown for the total series, with the number of mice used, the S.E., t and P values for the difference.

Table II*b* compares the differences in blood content of the tumour ascites between the three main groups for the total series, giving the number of mice used, the S.E., t and P values for the difference. The difference between groups I and II (2·583 per cent) is significant ($0{\cdot}05 > P > 0{\cdot}02$), while that between groups I and III (3·184 per cent) is highly so ($0{\cdot}01 > P > 0{\cdot}001$). The difference between groups II and III (0·7416 per cent) is not significant.

The relationship between the survival time and the amount of blood in the tumour ascites in the three main groups is shown in Table III, which gives the

TABLE III.—*The Relationship Between the Survival Time and the Blood Content of the Tumour Ascites in the Three Groups*

Group	Series	n	r	S.E.r	t	P
I	♂+♀	30	−0·8381	0·2397	3·496	0·01>P>0·001
	♂	15	−0·9831	0·2673	3·678	
	♀	15	−0·8708	0·2673	3·258	
II	♂+♀	29	−0·5125	0·1889	2·713	0·02>P>0·01
	♂	14	−0·4658	0·2773	1·680	0·2>P>0·1
	♀	15	−0·7591	0·2673	2·840	0·02>P>0·01
III	♂+♀	30	−0·01574	0·2397	0·06567	P>0·9
	♂	15	−0·02207	0·2673	0·08258	
	♀	15	−0·2633	0·2673	0·9851	0·4>P>0·3

The number of mice in each group and series is shown, and the correlation coefficient (r) with its S.E., t and P values.

number of mice in each group and series, the correlation coefficient (r), with its S.E., t and P values. In the control group I the amount of blood in the tumour ascites decreased with survival time. This negative correlation (−0·8381, −0·9831, −0·8708 for the total, male and female series, respectively) was highly significant in all cases ($0{\cdot}01 > P > 0{\cdot}001$). These findings are in keeping with those in the

author's previous experiment (Hartveit, 1961). In the vitally stained group II the negative correlation was greatly reduced ($-0{\cdot}5125$, $-0{\cdot}4658$, $-0{\cdot}7591$, respectively), being significant for the total and female series ($0{\cdot}02 > P > 0{\cdot}01$). It was even further reduced in the cortisone treated group III ($-0{\cdot}01574$, $-0{\cdot}02207$, $-0{\cdot}2633$) and as such was not significant.

With regard to the control groups 1, 2 and 3 ; none of the mice in group 1 died. One of the female mice in group 2 died 12 days after its first injection of trypan blue. One male and one female mouse in group 3 died after 21 injections of cortisone.

DISCUSSION

In this experiment there are three factors to be considered : the tumour, the relationship of the mice to the tumour and the interrelationship of the mice themselves.

As regards the tumour, Klein and Révész (1953) have shown that provided a sufficient tumour cell dose is used Ehrlich's ascites carcinoma can be expected to grow progressively, and the mouse to die within a time limit dependent on that tumour cell dose. The tumour cell dose used in the present experiment is comparable to that used in the author's previous experiment (Hartveit, 1961). The adult mice were taken from the same closed colony. On comparing the mice in group I of the present experiment to those in the previous experiment it is found that the actual difference in their mean survival time (0·53 days) is not significant ($0{\cdot}5 > P > 0{\cdot}4$). The difference in the mean blood content (1·7 per cent) is not significant either ($0{\cdot}2 > P > 0{\cdot}1$). In both cases the negative correlation between the survival time and the blood content of the tumour ascites is highly significant. Thus, although the tumour came from different transplant generations, there was no significant difference in the survival time or blood content, and the relationship between these two factors was unchanged. These facts support the view that the tumour can, in this case, be regarded as a relatively constant factor. In the present experiment all the tumour used came from one mouse, and this is an additional safeguard to the assumption that the tumour is a constant.

The mice were, of necessity, of different genetic make-up to the tumour, as Ehrlich's ascites carcinoma came originally from a heterozygous mouse (Snell, 1953). Therefore, one would expect that the mice would not accept transplants. But Ehrlich's ascites carcinoma is one of the so-called " non-specific " tumours that is said to contain fewer antigens than usual and so is less demanding than most in its choice of host (Barrett, 1958). However, a perfect fit between host and tumour cannot be expected. While the fit may be close enough to allow the tumour to grow the difference may show up in other ways—for example in the stromal reaction.

Thus, although the mice were not, as in Barrett's experiment referred to earlier (Barrett, 1942), sensitized beforehand with a foreign antigen, the tumour may possess tissue antigens that the mice lack. If they then react against this foreign protein their immunological response might be expected, on the basis of Barrett's findings, to be accompanied by haemorrhage into the tumour, i.e. by a change in the stromal reaction.

This hypothesis is based on the assumption that resistance to homotransplants is genetically determined. This has been shown to be true in the case of red blood cells (Cushing and Campbell, 1957) and normal tissues (Loeb and Wright, 1927 ;

Billingham, Brent, Medawar and Sparrow, 1954). The Mendelian nature of the genetic influences determining susceptibility to transplanted tumours has also been established (Little, 1956).

When a tumour is transplanted the tumour cells reproduce, the stroma is supplied by the host (Muir, 1951). In the case of Ehrlich's ascites carcinoma the stroma is represented by the ascitic fluid, which contains a variable number of white blood cells. In addition, in some cases, this stroma contains large numbers of red blood cells (Loewenthal and Jahn, 1932 ; Kun, Talalay and Williams-Ashman, 1951).

These red blood cells have appeared in the stroma either in direct response to the tumour cells, or in response to the mouse's reaction to these cells. If the first is the case we would expect that the amount of blood in the stroma would be constant for a given tumour cell dose. But the author has previously shown (Hartveit, 1961) that the amount of blood varies inversely with the survival time in mice receiving the same tumour cell dose. If the second is then true, the amount of blood should vary in accordance with the genetic dissimilarity between the mouse and the tumour.

Genetic dissimilarity is evidenced as resistance to transplantation (Snell, 1957). It has been shown that this resistance can be abrogated (Ludford, 1931 ; Andervont, 1936). The mice in the present experiment were treated in such a way as to abrogate their resistance to the transplantation of foreign tissue.

On the basis of the above reasoning one would expect that by supressing host resistance one would change the amount of blood present in the stroma : an increase indicating that the blood was an expression of the mouse's lack of resistance to the tumour, a decrease that it was an expression of its resistance. On the basis of Barrett's experiment (Barrett, 1942), one would expect a decrease.

The mice used were heterozygous. This does not affect their individual relationship to the tumour as discussed above, but different degrees of reaction are to be expected—those nearer to the tumour gentically reacting less against it. As the mice were from the same closed colony it is reasonable to assume that the genetic range in the three samples should be similar, and the results following abrogation of their natural resistance comparable.

All the mice receiving Ehrlich's ascites carcinoma in the present experiment were given the same dose of viable tumour cells. The dose used was chosen as it was known to give an ascitic tumour and a relatively short survival time. The dosage of trypan blue was based on that used by Andervont (1936), and the cortisone dosage on that used by Hobson (1960).

The results in the control mice show that the subcutaneous treatment alone will not influence the survival times of the experimental groups to any appreciable extent. Only one of the mice receiving trypan blue died during the course of the experiment and two of those given cortisone died towards its end.

The results in groups I, II and III show that, although all the mice died, their survival times and the blood content of their tumours differed considerably. Trypan blue did not alter the survival time of the mice but it did reduce the amount of blood in the tumour ascites. Cortisone increased the survival time of the female mice, and greatly reduced the amount of blood in the ascites in both sexes. In addition trypan blue, in part, and cortisone completely, abolished the negative correlation between the survival time and the amount of blood in the tumour ascites that was so strongly apparent in the control group.

Thus, by using methods claimed to reduce the natural resistance of the mice to tumour transplantation we have succeeded in increasing their survival time and decreasing their stromal reaction to the tumour. The blood content of the tumour must, therefore, be considered as an expression of resistance to Ehrlich's ascites carcinoma. We are then left with the paradoxical situation in which the mice with the greatest and most rapid local reaction to the tumour—indicating a type of resistance—die before those without such resistance. Those dying early died as a result of their immunological reaction to the homotransplant, while those surviving longer died as a result of their acceptance of the tumour that was able to grow progressively until it killed the host mechanically.

This finding that the blood content of the tumour is an expression of the resistance of the mouse to the tumour may be of practical use in the field of tumour immunity. In 1958 Barrett stated that antibodies " have eluded detection " in this field. Since then *in vitro* tests for haemagglutinins (Feldman and Sachs, 1957), the tanned erythrocyte technique (Finney, Byers and Wilson, 1960), complement fixation (Lund, 1958) and skin tests (Grace and Dao, 1958) have been reported to give positive results. However, most evidence of such immunity is circumstantial. Thus when an animal is immunized against a tumour the test of whether immunity has been produced is whether or not a transplant of that tumour will be able to grow in the host. Other methods involve the use of an immune serum from a foreign host (Flax, 1956). In this case the antibody is an antibody to a foreign protein, and is not dependent on the fact that the cells were tumour cells. With Ehrlich's ascites carcinoma the amount of blood in the ascites may prove to be a yard-stick by which it will be possible to measure induced as well as natural immunity to the tumour. Experiments in this direction are in progress.

It may be that this haemorrhagic reaction is a Shwartzman-like phenomenon. It has been suggested that the Shwartzman phenomenon may be a manifestation of an immune response (Thomas, 1954). Stetson (1955) has shown that injection of bacterial endotoxin can elicit haemorrhage in skin areas previously prepared by the intradermal injection of homologous or heterologous bacterial products, and compares the reaction to that following the injection of tuberculin in specifically sensitized rabbits. He postulates that both reactions involve a delayed type of allergy, that is accompanied by focal (haemorrhagic) reactions, and systemic reactions—following intraperitoneal injection of the endotoxin—that may be fatal. Lawrence (1956) has drawn analogies between this type of delayed sensitivity and homograft rejection. In the present experiment it has been shown that the haemorrhagic response occurring in some of the mice following transplantation of the tumour is dependent on their natural resistance to the transplant. This response can be likened to the Shwartzman phenomenon in that the mice were previously sensitive to the transplant and showed focal haemorrhage, and a severe systemic reaction leading to death, following a massive intraperitoneal dose of the incompatible material.

SUMMARY

Three groups of 30 heterozygous mice were injected with Ehrlich's ascites carcinoma. Two of the groups of animals were treated with trypan blue and cortisone, respectively, in an attempt to abrogate their natural resistance to the tumour. The amount of blood in the tumour ascites was found to be less following these treatments. (Average per cent : Control—4·3, trypan blue—1·7, cortisone—1·2).

Thus the blood content of the tumour ascites can be regarded as an expression of the animal's reaction against, i.e. natural resistance to, the tumour. It was also shown that the survival time of the mice that did not react was greater than that of those which reacted against the tumour—suggesting that the latter died as a result of their immunological reaction to an overwhelming dose of foreign tissue. It is possible that this reaction is comparable to a Shwartzman-like phenomenon.

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Made and printed in Great Britain for
H. K. Lewis & Co. Ltd., by
Adlard & Son, Ltd., Bartholomew Press, Dorking.

Reprinted from
The British Journal of Cancer.
1962. Vol. XVI, p. 323

EVIDENCE OF INDUCED IMMUNITY TO EHRLICH'S ASCITES CARCINOMA BROUGHT ABOUT THROUGH THE COMBINATION OF FREUND'S ADJUVANT WITH LIVING TUMOUR

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Received for publication February 27, 1962

In this experiment living Ehrlich ascites carcinoma was combined with Freund's adjuvant in an attempt to induce immunity to the tumour. This adjuvant has been shown to enhance antibody production and to produce a specific allergic type response to hetero-, homo-, and autotransplants (Freund, 1956; Witebsky, Rose, Paine and Egan, 1957; McMaster, Lerner and Exum, 1961). Both serum antibodies and delayed sensitivity have been reported following its use (Freund, 1956). Medawar (1948) has shown that delayed sensitivity is involved in the rejection of tissue transplants, and it is also involved in the rejection of tumour transplants (Lawrence, 1956).

It has been shown that a combination of dead tumour in Freund's adjuvant may result in the production of tumour antibodies (Fink, Smith and Rothlauf, 1955; Witebsky, Rose and Shulman, 1956; Graham and Graham, 1959; Finney, Byers and Wilson, 1960), but these are not easy to demonstrate. In the present experiment living tumour was used to avoid the possibility of denaturating the tumour protein, and subcutaneous injection was chosen as it is thought to be optimal for the development of delayed sensitivity (Lawrence, 1956). The blood content of the tumour ascites was used as a measure of the tumour immunity (Hartveit, 1961*b*).

MATERIAL AND METHODS

Mice.—These were similar to the mice used in previous experiments (Hartveit, 1961*b*) and were selected in the same way. Eight groups of 15 males and 15 females were set up.

Tumour.—The Ehrlich ascites carcinoma used was taken from the 84th transplant generation of the tumour maintained at this Institute.

Preparation of the adjuvant.—Freund's method (1956) for the preparation of complete adjuvant was followed. Eucerin anhydrous was used as the emulsifying agent. The tubercle bacilli were of strain H37, Rv (kindly supplied by Professor Vogelsang, Gade Institute, Department of Microbiology). Four ml. of this adjuvant mixture were emulsified with 4 ml. of saline.

Preparation of tumour inoculum.—The tumour ascites from two male and two female mice that had been injected intraperitoneally with Ehrlich's ascites carcinoma 10 days previously, was pooled. The resulting fluid contained 2,110,000 tumour cells/mm^3; PCV 31 per cent, and 1 per cent packed erythrocytes. All the tumour cells were considered viable as none stained by Schrek's method (1936). Four ml. of this ascites were emulsified with 4 ml. of the adjuvant mixture. A

further quantity was diluted with saline to give the same tumour cell dose/unit volume as in the tumour-adjuvant mixture. The remaining fluid was used undiluted.

Experimental procedure.—The treatment given to the mice is summarized in Table I. The intraperitoneal tumour dose was 0·1 ml. The subcutaneous injections (0·05 ml.) were given on the back. When the injections had been completed only an occasional tumour cell in the remaining fluid was of doubtful viability.

TABLE I.—*The Treatment given to the Mice in the Different Groups.* (*EAC = Ehrlich's ascites carcioma. FA = Freund's adjuvant*)

Group	Route of injection: Subcutaneous	Route of injection: Intraperitoneal
1	nil	nil
2	nil	EAC
3	FA + EAC	nil
4	FA + saline	nil
5	EAC + saline	nil
6	FA + EAC	EAC
7	FA + saline	EAC
8	EAC + saline	EAC

Thereafter any subcutaneous growths were measured weekly. When a mouse died the survival time was recorded in days. If a subcutaneous growth was present this was measured. If the mouse had been given an intraperitoneal injection 1 ml. of the ascites was centrifuged and the PCV of the erythrocytes recorded (Hartveit, 1961*a*).

RESULTS

Survival time.—Table II gives the mean survival time of the mice that received intraperitoneal tumour. The differences between the groups are not significant for the total or male series. The females in groups 6 and 7 died significantly earlier than those in group 8 ($0{\cdot}05 > P > 0{\cdot}02$ and $0{\cdot}01 > P > 0{\cdot}001$, respectively). The females in group 7 also died earlier than the males in the same group ($0{\cdot}01 > P > 0{\cdot}001$). The sex difference in the other 3 groups is not statistically significant.

All the mice in the remaining control groups were alive a week after the last mouse with intraperitoneal tumour died ; showing that the mice themselves and their surroundings were healthy, and that the subcutaneous treatment did not kill the mice within the time limit of the experiment. The survival times of these animals will be discussed in a later paper.

TABLE II.—*The Mean Survival Time (with SD) of the Mice in the Groups with Intraperitoneal Tumour.* (*30 mice in each group*)

Group	Mean survival time (days): Total series	Male series	Female series
2	12·8 ± 2·944	13·86 ± 3·178	11·73 ± 2·220
6	11·7 ± 3·435	12·8 ± 3·661	10·6 ± 2·846
7	11·93 ± 2·497	13·26 ± 2·657	10·6 ± 1·342
8	12·9 ± 1·817	13·06 ± 1·378	12·73 ± 2·168

The blood content of the tumour ascites.—This was measured in groups 2, 6, 7 and 8, and the mean percentages are shown in Table III. The greatest amount of blood was present in group 6. Groups 2 and 7 showed approximately the same amount for the total series, while group 8 showed least. The difference between groups 6 and 8 is significant for the total and female series ($0{\cdot}01 > P > 0{\cdot}001$ and $0{\cdot}02 > P > 0{\cdot}01$, respectively). The sex difference was only significant in group 7 in which the males showed less blood than the females ($0{\cdot}001 > P$). As a result of this the difference in blood content between the males in groups 6 and 7 becomes significant ($0{\cdot}05 > P > 0{\cdot}02$).

TABLE III.—*The Mean Blood Content of the Ascites (with SD) in the Mice with Intraperitoneal Tumour.* (30 *mice in each group*)

	Mean blood content (per cent)		
Group	Total series	Male series	Female series
2	5·1 ± 2·902	4·26 ± 2·755	5·93 ± 2·795
6	6·931 ± 4·842	6·0 ± 4·957	7·8 ± 4·564
7	5·0 ± 2·532	3·071 ± 1·594	6·8 ± 1·797
8	3·86 ± 2·565	3·4 ± 2·184	4·3 ± 2·802

The results in male groups 6 and 7 are based on 14 mice as too little ascites was present in the remaining two.

The correlation between the survival time and the blood content of the ascites (groups 2, 6, 7 and 8).—Table IV shows that this negative correlation is highest in the control group 2 ($0{\cdot}001 > P$), and next highest in group 6 ($0{\cdot}001 > P$), the experimental group. It is statistically significant in all groups but group 7.

TABLE IV.—*Correlation Between Survival Time and Blood Content of the Ascites in Mice with Intraperitoneal Tumour.* (30 *mice in each group*)

	Correlation coefficient (r)		
Group	Total series	Male series	Female series
2	−0·8383	−0·8774	−0·7700
	($0{\cdot}001 > P$)	($0{\cdot}01 > P > 0{\cdot}001$)	($0{\cdot}02 > P > 0{\cdot}01$)
6	−0·7905	−0·7900	−0·7844
	($0{\cdot}001 > P$)	($0{\cdot}02 > P > 0{\cdot}01$)	($0{\cdot}02 > P > 0{\cdot}01$)
7	−0·6195	−0·2505	−0·5542
	($0{\cdot}01 > P > 0{\cdot}001$)	($0{\cdot}4 > P > 0{\cdot}3$)	($0{\cdot}1 > P > 0{\cdot}5$)
8	−0·6653	−0·7638	−0·5981
	($0{\cdot}01 > P > 0{\cdot}001$)	($0{\cdot}02 > P > 0{\cdot}01$)	($0{\cdot}05 > P > 0{\cdot}02$)

Results based on 14 male mice in groups 6 and 7.

For the purposes of the following calculations the mean survival time of the animals in group 2 (12·8 days) was taken as the dividing line between those with a short (a) and those with a long (b) survival time following intraperitoneal injection of the tumour.

The distribution of the survival time of the mice within the groups (2, 6, 7 and 8).—Table V shows that the mice in groups 2 and 8 were more or less evenly distributed between groups a and b, while group 7 showed more, and group 6 even more, mice in group a. In groups 2 and 8 the sex difference in this distribution is not signi-

ficant, while it is so in groups 6 and 7 ($0{\cdot}01 > P > 0{\cdot}001$) as many more females than males fall into group a. For the total series the difference in the percentage of mice in group a is significant between groups 6 and 8, and for the females between groups 6 and 8 ($0{\cdot}01 > P > 0{\cdot}001$) and 7 and 8 ($0{\cdot}05 > P > 0{\cdot}02$).

TABLE V.—*The Distribution of the Survival Time of the Mice with Intraperitoneal Tumour within the Groups, giving Number of Mice (per cent)*

	Survival time					
	Short (a)			Long (b)		
Group	Male	Female	Total	Male	Female	Total
2 .	5	10	15 (50)	10	5	15 (50)
6 .	7	14	21 (72·4)	7	1	8 (27·6)
7 .	5	13	18 (62·1)	9	2	11 (37·9)
8 .	6	8	14 (46·7)	9	7	16 (53·3)

The results in male groups 6 and 7 are based on 14 instead of 15 mice as the ascites in the remaining two was not suitable for further investigation.

The distribution of the mean blood content of the tumour ascites (per cent) according to the survival time within the groups (2, 6, 7 and 8).—Table VI shows that in all cases there was more blood in group a than b. This difference was statistically significant in all groups. The sex difference within the groups was significant in group 7a ($0{\cdot}001 > P$), and in group 6b (but the latter contains only one female mouse). For the total series the difference between groups 6a and 8a is significant ($0{\cdot}01 > P > 0{\cdot}001$), for the male series that between groups 6a and 7a, and for the females those between groups 2b and 6b and 2b and 8b ($0{\cdot}05 > P > 0{\cdot}02$).

TABLE VI.—*The Distribution of the Mean Blood Content of the Tumour (per cent), with SD, according to the Survival Time within the Groups of Mice with Intraperitoneal Tumour.* (30 *mice in each group*)

	Survival time					
	Short (a)			Long (b)		
Group	Male	Female	Total	Male	Female	Total
2 .	$6{\cdot}8 \pm 3{\cdot}763$	$6{\cdot}9 \pm 2{\cdot}468$	$6{\cdot}86 \pm 3{\cdot}891$	$3{\cdot}0 \pm 1{\cdot}673$	$4{\cdot}0 \pm 2{\cdot}408$	$3{\cdot}3 \pm 2{\cdot}037$
6 .	$8{\cdot}7 \pm 5{\cdot}719$	$8{\cdot}36 \pm 4{\cdot}201$	$8{\cdot}47 \pm 4{\cdot}659$	$3{\cdot}29 \pm 1{\cdot}375$	$0{\cdot}0 \pm 0{\cdot}0$	$2{\cdot}9 \pm 1{\cdot}458$
7 .	$3{\cdot}4 \pm 1{\cdot}908$	$7{\cdot}23 \pm 1{\cdot}480$	$6{\cdot}17 \pm 4{\cdot}078$	$2{\cdot}89 \pm 1{\cdot}485$	$4{\cdot}0 \pm 1{\cdot}0$	$3{\cdot}09 \pm 1{\cdot}475$
8 .	$4{\cdot}0 \pm 1{\cdot}965$	$5{\cdot}62 \pm 2{\cdot}287$	$5{\cdot}0 \pm 2{\cdot}3$	$2{\cdot}89 \pm 5{\cdot}509$	$2{\cdot}86 \pm 2{\cdot}641$	$2{\cdot}88 \pm 2{\cdot}367$

The results in male groups 6 and 7 are based on 14 mice as too little ascites was present in the remaining two.

The subcutaneous injection site.—This was examined in all appropriate groups on the 7th day after injection. The findings are shown in Table VII. The three mice in group 6 that had died by this time showed thickening under the skin at the injection site, but no palpable masses. The differences in mean tumour diameter between groups 3, 5 and 8 are not statistically significant. Thus the addition of adjuvant to the tumour has not affected its vitality. The difference between groups 3 and 6 (Fig. 1), is significant ($0{\cdot}001 > P$). The sex difference is only significant in group 5 in which the tumours in the males were larger than those in the females ($0{\cdot}05 > P > 0{\cdot}02$).

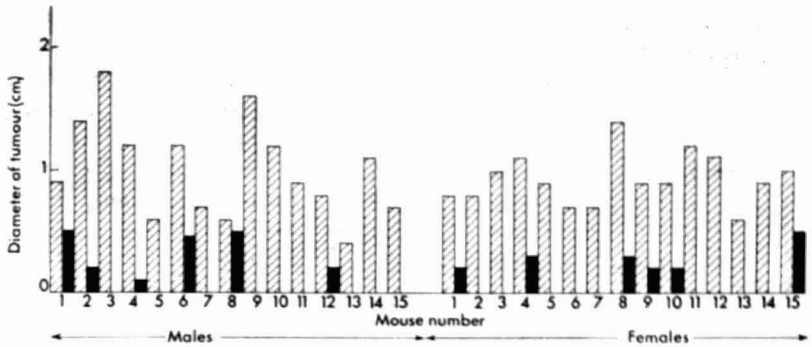

FIG. 1.—Greatest subcutaneous tumour diameter 7 days after injection in group 3 (hatched columns) and group 6 (dark columns). Values for individual mice in each group given alternately. Note: The tumours were measured at autopsy in 3 mice in group 6.

TABLE VII.—*The Average Greatest Diameter (mm) of the Subcutaneous Tumours (with SD) at 7 Days*

Group	Tumour diameter (mm.)	SD (mm.)
3	9·73	2·112
5	9·166	3·976
6	1·4	1·698
8	7·13	4·249

All the mice that had been given adjuvant without tumour subcutaneously (groups 4 and 7) showed slight thickening at the injection site on the 7th day. At 14 days in group 4, and at 14 days or at autopsy in group 7, the site was difficult to locate in the males while all the females showed a distinct freely movable swelling (approximately 0·5 cm. in diameter) in the subcutaneous tissues. Ulceration did not occur.

DISCUSSION

The majority of attempts to detect serum antibody to tumour tissue have failed (Barrett, 1958) and some of the serum antibodies that have been reported (Southam, 1960) may have been due to genetic differences between host and tumour and not to the fact that the tissue was tumour tissue. For there to be a genetic difference between a host and its own tumour a mutation must have occurred. The mutation, in altering the function of the mutant gene (Oglinsky and Umbreit, 1959), may result in a change in the protein synthesised. This protein may then be recognized as " non-self " : a change in a surface protein producing serum antibody, one deep in the cell giving delayed sensitivity. But if the mutation results in a change in the rate of protein synthesis leaving the structure unchanged (Brinkhaus and Graham, 1954 ; Pitney and Elliott, 1960), no recognition can be expected. This, or a change in an intracellular protein, could explain the almost universal failure to detect serum antibody to autologous tumours. In the former case any enhancing action shown by Freund's adjuvant could be due to the mobilization of immunologically active cells (Burnet, 1959). In the latter some factor in the adjuvant may combine with the " self " protein, the combination being recognized immunologically (Freund, 1956). Such haptene

formation is thought to occur in the autoimmune diseases (Favour, 1956 ; Voisin, Toullet and Maurer, 1958 ; Asherson and Broberger, 1961).

In reactions involving normal autologous tissues the second mode of action must be postulated as the possibility of genetic difference does not exist. While reaction against the protein-haptene complex could be expected, reaction against the original protein has, in fact, been demonstrated (McMaster, Lerner and Exum, 1961). Thus, it is not necessary to postulate a genetic difference between host and tumour to explain the rationale of using a combination of autologous tumour and Freund's adjuvant in an attempt to produce changes in a distant transplant of the tumour.

While the tumour used in this experiment is a homograft it is known that some of the mice used will accept it as an autograft, the blood content of the tumour ascites being a measure of their natural immunity (Hartveit, 1961*b*). The present experiment was designed to see if subcutaneous treatment with Freund's adjuvant combined with living tumour would increase the immunological response of the mice to the tumour and, if this were so, whether the increase was in natural or acquired resistance. To show this the distribution of the mice within the groups was investigated. The mean survival time of the animals in the control group 2 was taken as the dividing line between those with a short (a) and those with a long survival time (b) ; the mice in group a being those resisting the tumour, group b accepting it. If the treatment resulted in a greater number of mice in group a than could be expected on the basis of the findings in the controls—it would then have been shown that immunity had been induced in some of the mice that would normally have accepted the tumour without response.

The results show that the mice in group 8 had the longest survival time, the least blood in the tumour ascites and the highest number of mice in group b. Thus these mice show most evidence of accepting the tumour homograft, even more than the untreated control group 2. This may be an example of an XYZ effect (Casey, Hatherway and Casey, 1956 ; Goldie, Walker, Kelley and Gaines, 1956) brought about through a decrease in the immunological response of the host. This is in keeping with clinical experience of malignant growths (Graham and Graham, 1955). As the mice in this group show least evidence of natural immunity they are, in fact, more suitable than group 2 as a control for the experimental group 6.

The following differences between group 6 and 8 are statistically significant. The females in group 6 died earlier than those in 8. There was more blood in the ascites in group 6 than in group 8. Group 6 showed a higher percentage of mice in group a than did group 8, and there was a higher blood content in the ascites in 6a than 8a.

These results consistently indicate that the subcutaneous tumour-adjuvant mixture increased the immunological response of the mice to the intraperitoneal tumour. The effect is particularly marked in the females in group 6 only one of which remained in group b, i.e. was unaffected by the adjuvant-antigen mixture. The increase in the tumour blood content shows that the immunity of the mice which already possess some natural resistance has been strengthened. The increase in the number of mice in group a shows that immunity has been induced in some mice that previously lacked resistance. As it has proved possible to measure both natural and acquired immunity through the blood content of the tumour it is probable that the same mechanism is at work in both cases.

As the mice in group 7 received adjuvant alone subcutaneously the decrease in survival time and increase in tumour blood content in the female mice must be regarded as non-specific. Non-specific allergic responses have been reported with normal tissues following the injection of adjuvant alone (Voisin, Toullet and Maurer, 1958), but a marked sex difference has not been described previously. This sex difference is further reflected in the reaction at the subcutaneous injection site, which was marked in the females and absent in the males. As the findings in group 4 were similar the reaction is not dependent on the presence of intraperitoneal tumour. It is often easier to produce specific tumour immunity in female mice (Gross, 1943) as is shown in group 6. It appears that non-specific immunity also is easier to produce in females.

This non-specific immunity must differ in some way from that produced by adjuvant combined with antigen as the normal relationship between the survival time and the tumour blood content, that holds in group 6, has been upset in group 7. It may be that the mode of action of adjuvant alone differs from that of an adjuvant-antigen mixture. While the adjuvant alone will be able to mobilize immunologically active cells, haptene formation will not be possible. This strengthens the idea that haptene formation is concerned in the adjuvant-antigen response to tumour tissue.

It was also found that in mice with intraperitoneal tumour, the tumour in the adjuvant-antigen mixture failed to grow as quickly as in the mice without a further source of tumour (Table VII and Fig. 1). This is contrary to what would be expected on the basis of an XYZ effect, and is difficult to explain. It may be that the intraperitoneal tumour stimulates the sensitivity reaction by neutralizing its products.

SUMMARY

Freund's adjuvant given subcutaneously in combination with living Ehrlich ascites carcinoma was found to increase the immune response of mice to the intraperitoneal injection of the same tumour. Both natural and acquired immunity appear to have been affected. An increase in the immune response was also found in female mice following Freund's adjuvant alone. Evidence is presented that the mechanism of this non-specific reaction differed from that of the specific reaction. The combination of adjuvant and living tumour had an inhibitory effect on the growth of the tumour in the adjuvant mixture when intraperitoneal tumour was also present.

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Made and printed in Great Britain for
H. K. Lewis & Co. Ltd., by
Adlard & Son, Ltd., Bartholomew Press, Dorking.

Reprinted from
The British Journal of Cancer,
1962, Vol. XVI, p. 331

FURTHER OBSERVATIONS ON THE RESULTS OF COMBINING FREUND'S ADJUVANT WITH LIVING EHRLICH ASCITES CARCINOMA

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Received for publication March 20, 1962

In a previous paper (Hartveit, 1962) the production of immunity to Ehrlich's ascites carcinoma by means of the subcutaneous injection of the same tumour combined with Freund's adjuvant was studied. In the course of that experiment some further points arose. Firstly, to what extent did the combination with Freund's adjuvant damage the tumour cells? This will be reflected in the survival time of the mice and is discussed in part (a) of this paper. Secondly, a marked non-specific immune reaction was noticed in female mice following the subcutaneous injection of adjuvant alone. Would this reaction be influenced by a time interval between the subcutaneous and the intraperitoneal injection? This is discussed in part (b). Thirdly, it was noticed that haemolysis occurred in the tumour ascites in some of the mice. This was not mentioned previously and will now be presented in detail in part (c) of this paper together with the corresponding findings in part (b).

It will be noted that the experimental material concerned here is that used in the previous experiment (Harveit, 1962). The composition of the material and the treatment given are summarized in Table I, which also shows the additional treatment given to the mice in groups 1 and 4 in the experiment reported in part (b) of this paper.

TABLE I.—*The Composition of the Experimental Material and the Treatment given in the Different Groups.* (15 *Male and* 15 *Female Mice in Each Group*)

Group	Route of injection: Subcutaneous (day 1)	Route of injection: Intraperitoneal
1	Nil	EAC (day 30)
2	Nil	EAC (day 1)
3	FA + EAC	Nil
4	FA + saline	EAC (day 30)
5	EAC + saline	Nil
6	FA + EAC	EAC (day 1)
7	FA + saline	EAC (day 1)
8	EAC + saline	EAC (day 1)

FA = Freund's adjuvant. EAC = Ehrlich's ascites carcinoma.

The following experiments were carried out :

(a) *The Survival Time of Mice following the Subcutaneous Injection of Ehrlich's Ascites Carcinoma and Freund's Adjuvant*

The two control groups with which we are concerned here are group 3, in which the mice were given a mixture of Freund's adjuvant and Ehrlich's ascites

carcinoma subcutaneously, and its control group 5 in which they were given tumour alone (Table I). Comparison of the survival time in these two groups should establish the extent to which the tumour was injured by its combination with Freund's adjuvant.

Material and methods

For a detailed description of these the reader is referred to the original paper (Hartveit, 1962) and to Table I of this paper. To summarize: Each group contained 15 male and 15 female mice. The mice in group 3 were each given one subcutaneous injection of a mixture of Ehrlich's ascites carcinoma and Freund's adjuvant, while those in group 5 each received one subcutaneous injection containing the same number of tumour cells as those in group 3. The tumour came from the same source for both groups and, with the exception of the emulsifying process used before injection in group 3, was handled in the same way.

The mice were then examined weekly for the presence of subcutaneous tumour and any growth present was measured. The survival time was recorded in days.

Results

Tumour nodules occurred at the site of the subcutaneous injection in all animals. At one week there was little difference in the mean tumour diameter in groups 3 and 5 (9·7 mm. $\pm$ 2·1 and 9·2 mm. $\pm$ 3·9, respectively). By three weeks every tumour had reached at least 1 cm. in diameter (i.e. double the size of the nodules found on injection of adjuvant alone in females). Thereafter the tumours regressed in two animals in each group; in 2 females in group 3 and in 1 male and 1 female in group 5. These four mice were alive without sign of tumour one month after all the rest were dead. They were excluded from the survival time calculations. The remaining animals died with actively growing tumours.

Table II shows the mean survival time of these mice, with the SD of this mean, the SE of the difference between the female and the male means and its P value. It will be seen that the mean survival time of the animals of the same sex in both groups was approximately the same, and that the marked sex difference ($0{\cdot}001 > P$) occurred in both groups.

TABLE II.—*The Mean Survival Time in Days (x) of Mice with Subcutaneous Ehrlich Ascites Carcinoma and Freund's Adjuvant (Group 3) Compared to that of Mice with Subcutaneous Ehrlich Ascites Carcinoma Alone (Group 5), Giving the Sex and Number of the Mice, the Mean Survival Time and SD, the SE of the Sex Difference in Means and its P value*

Group	Sex	Number	$\bar{x}$	S.D.$\bar{x}$	S E. ($\bar{x}$♀ − $\bar{x}$♂)	P
3	♂	15	48·5	16·4	4·74	$0{\cdot}001 > P$
	♀	13*/*	78·7	24·4		
5	♂	14*	49·3	13·45	4·48	$0{\cdot}001 > P$
	♀	14*	80·3	31·65		

* One mouse survived, see text.

Discussion

The number of cells injured or killed during the emulsifying process undergone by the tumour cells in group 3 does not appear to have affected the survival time of the mice, which is almost identical to that of the controls which were given the same dose of untreated tumour. It may be, however, that a large majority of the cells were killed and that these dead cells exerted the well known XYZ effect (Casey, Hatherway and Casey, 1956) on those remaining. This could offset the reduced tumour cell dosage and bring the survival time back in line with that in group 5. In any case, the assumption made in the previous paper (Hartveit, 1962) that living Ehrlich ascites carcinoma was used in combination with Freund's adjuvant can be upheld.

The findings here show that the tumour cells in the adjuvant mixture continue to grow in the absence of a further source of tumour. In the previous experiment (Hartveit, 1962) it was found that the tumour cells in the same adjuvant mixture did not grow in the presence of intraperitoneal tumour. This rules out the prophylactic use of such a mixture while, at the same time, it indicates that the mixture may be of some use in the treatment of a tumour-bearing host.

It is of note that there was a marked sex difference in the survival time in both groups. This is contrary to the author's findings with interaperitoneal tumour (Hartveit, 1961*a*), and contrary to the general experience of the intraperitoneal growth of this tumour. It is likely that the rapid intraperitoneal growth rate prevents the sex difference, which becomes apparent following the slower subcutaneous growth, from showing up. Thus female mice, in which it is easier to induce tumour immunity (Gross, 1943), also have a greater natural immunity to the Ehrlich ascites carcinoma than male animals.

(b) *The Non-specific Action of Freund's Adjuvant on Ehrlich's Ascites Carcinoma*

In the course of the main experiment (Hartveit, 1962) it was found that female mice given a subcutaneous injection of Freund's adjuvant alone on the same day as an intraperitoneal injection of Ehrlich's ascites carcinoma showed a decrease in survival time and an increase in the blood content of their tumours when compared to male mice given the same treatment. This reaction must be considered to be non-specific in contrast to that produced by the subcutaneous injection of Freund's adjuvant plus living tumour. It is suggested in the above mentioned paper that the latter reaction is mediated through the same channels as that due to natural immunity, while the former clearly operates in a different way. It was also found that the female mice showed a marked reaction at the subcutaneous injection site while this was absent in the males.

The sex differences in the responses mentioned above were highly significant statistically. It was decided to follow up these findings to see if a time interval between the injection of the subcutaneous adjuvant and that of the intraperitoneal tumour would affect the reactions. A new experiment was, therefore, undertaken in which mice that had received Freund's adjuvant and saline subcutaneously were given intraperitoneal tumour 30 days later, instead of on the same day as in the main experiment (Hartveit, 1962).

Material and methods

The reader is referred to the previous paper (Hartveit, 1962) and to Table I of this paper for details. To summarize : There were 15 male and 15 female mice

in each group. Group I was the untreated control group while group 4 received Freund's adjuvant subcutaneously. Thirty days after the mice in group 4 had been injected with adjuvant all the mice in both groups were given an intraperitoneal injection of 0·1 ml. of Ehrlich's ascites carcinoma (2,300,000 tumour cells/c.mm.). This tumour was taken from a mouse of the 87th transplant generation. The tumour used in the previous experiment had come from the 84th transplant generation of the same tumour.

At the time of the injection of the intraperitoneal tumour the subcutaneous swellings (approximately 0·5 cm. diameter), that had appeared within 14 days of the injection of the adjuvant mixture in the females, persisted in all cases. In the males the site was difficult to locate.

When the mice died the survival time was recorded in days and the blood content of the tumour recorded after centrifugation, as described in the previous paper (Hartveit, 1962). The subcutaneous injection site was examined at autopsy in group 4.

Results

Table III shows the mean survival time (days), plus the SD of the mean, of the mice in both groups. The female mice in the experimental group 4 showed little difference in survival time from those in the control group 1. On the other hand, there was a marked difference in the survival time of the male mice ; those in group 4 dying before the controls (13·64 and 17·4 days, respectively). This difference is statistically significant ($0{\cdot}05 > P > 0{\cdot}02$).

TABLE III.—*The Mean Survival Time (Days), plus SD, of Mice given Intraperitoneal Ehrlich's Ascites Carcinoma 30 days after Subcutaneous Freund's Adjuvant (Group 1) and of Controls lacking Subcutaneous Adjuvant (Group 4)*

Group	Sex	Number of mice	Mean survival time	SD
1	♂	15	17·4	6·332
	♀	15	12·3	3·194
4	♂	14*	13·64	2·811
	♀	15	12·6	4·775

* One mouse accidentally killed.

Table IV gives the mean blood content of the tumour (per cent), plus the SD of the mean, in both groups. Once again there is little difference in the results in female mice, while the blood content in the males in the experimental group 4 is

TABLE IV.—*The Mean Blood Content (per cent), Plus SD, of the Intraperitoneal Tumour in Groups 1 and 4*

Group	Sex	Number of mice	Mean blood content	SD
1	♂	14†	3·143	2·530
	♀	15	5·2	4·651
4	♂	13*†	5·0	2·037
	♀	15	5·266	3·347

* One mouse accidentally killed.
† Tumour unsuitable for investigation in one mouse.

much higher than in the controls (5 and 3·14 per cent, respectively). This difference is significant statistically ($0{\cdot}05 > P > 0{\cdot}2$).

Table V shows the correlation between the survival time and the tumour blood content (correlation coefficient, r) for both sexes in both groups. While the negative correlation is high in the females in both groups, it is much lower in the males in group 4 than in group 1.

TABLE V.—*The Correlation between Survival Time and Tumour Blood Content in Groups* 1 *and* 4, *using the Correlation Coefficient r*

Group	Sex	Number of mice	*r*
1	♂	14†	−0·5163
	♀	15	−0·8501
4	♂	13*†	−0·1642
	♀	15	−0·8027

* One mouse accidentally killed.
† Tumour unsuitable for investigation in one mouse.

At autopsy the subcutaneous injection site was easily located in 14 out of the 15 females as the subcutaneous swelling had persisted. It could not be detected macroscopically in the males.

Discussion

It has previously been shown that the survival time of mice with intraperitoneal Ehrlich ascites carcinoma is shorter in those that show a sensitivity reaction to the tumour, and that the blood content of the tumour ascites is a measure of this reaction, which is probably one of rejection of the foreign tumour protein (Hartveit, 1961*b*). Thus it provides a measure of the animal's natural immunity to the tumour. In addition there is also evidence that it can be used to measure acquired immunity (Hartveit, 1962).

In the latter experiment it was found that female mice showed this immunity reaction if they were given a subcutaneous injection of Freund's adjuvant without antigen on the same day as the intraperitoneal injection of Ehrlich's ascites carcinoma. This was unexpected as Freund (1956) clearly states that, " the adjuvant remains without effect if injected into a separate area ", i.e. if antigen is not combined with adjuvant. On the other hand, Voisin, Toullet and Maurer (1958) have reported non-specific testicular lesions following the use of adjuvant alone.

In the present experiment the adjuvant that was given subcutaneously in group 4 was the same as that used in group 7 of the main experiment (i.e. the mice that received subcutaneous adjuvant on the same day as they were given intraperitoneal tumour). The tumour used was taken from the same source 3 transplant generations later. Thus conditions in the two experiments are strictly comparable—the time limit between the subcutaneous and intraperitoneal injections being the determining factor.

The results show that it is now the male animals that are responding to the adjuvant injection—the females reacting in the same way as the controls. The

mean survival time of the males is now significantly less than that of the control males (13·64 days compared to 17·4 days), and the blood content of their tumours greater (5 per cent compared to 3·14 per cent). In addition the degree of correlation between the survival time and the tumour blood content is greatly reduced (−0·1642 compared to −0·5163). The subcutaneous injection sites did not show any change.

The mean survival time of the females, the blood content of their tumours and the degree of correlation between these two factors now appears to be unchanged by the treatment.

These results show that the time interval in this case was important to the response of the mice. The immune reaction of the females developed shortly after the injection of the adjuvant, while that of the males became evident later. In the main paper (Hartveit, 1962) it was suggested that the reaction in the females differed in mechanism from that due to natural immunity or immunity acquired after the subcutaneous injection of Freund's adjuvant and living Ehrlich ascites carcinoma, as the negative correlation between the survival time and the tumour blood content, that is normally found in these mice, was upset by the treatment. The present results support this idea as once more the animals responding to the subcutaneous injection of adjuvant alone (now only the males) show the same lack of correlation between these two factors. The experiment also shows that the immune reaction is not dependent on the reaction at the subcutaneous injection site.

(c) *Haemolysis in the Ehrlich Ascites Carcinoma following the Subcutaneous Injection of Whole Tumour Fluid plus Freund's Adjuvant*

In the course of the analysis of the results of the main experiment (Hartveit, 1962) and of part (b) of this paper, it came to light that there were marked differences in the amount of haemolysis in the ascitic fluid in the various groups. These findings will now be presented in detail.

Material and methods

For a full description of these the reader is referred to the original paper (Hartveit, 1962), and to part (b) and Table I of this paper. To summarize : All groups contained 15 male and 15 female mice. The experimental group 6 was given a mixture of Freund's adjuvant and living Ehrlich ascites carcinoma subcutaneously, while groups 1 and 2 had no subcutaneous treatment. Groups 4 and 7 were given subcutaneous adjuvant minus tumour ; group 8 subcutaneous tumour alone. On the first day of the experiment the mice in groups 2, 6, 7 and 8 each received intraperitoneal Ehrlich ascites carcinoma. Thirty days later groups 1 and 4 were given intraperitoneal tumour. The remaining groups are not concerned in this experiment.

When the mice died the tumour ascites was removed and centrifuged as described previously. After centrifugation the haemolysis in the supernatant fluid was graded :

0 = absent, pale yellow supernate.
\+ = present, pink supernate.
++ = marked, dark red supernate.

Results

The findings in both the males and the females are given in Table VI which shows that ++ haemolysis occurred only in groups 6, 7 and 4 (in 30 per cent, 3·3 per cent and 3·6 per cent respectively). The difference in this percentage between group 6 and the untreated mice (group 2) is highly significant statistically ($0{\cdot}01 > P > 0{\cdot}001$). There were no marked sex differences.

TABLE VI.—*Haemolysis in the Ascitic Fluid of Mice with Intraperitoneal Ehrlich Ascites Carcinoma (EAC) following the Subcutaneous Injection of Living EAC plus Freund's Adjuvant (FA), and in Control Groups.* (30 *Mice in Each Group*)

Group	Treatment	Sex	Haemolysis+ 0	+	++	%++
2	Nil	♂	10	5	0	0
		♀	10	5	0	
6	EAC + FA	♂	8	3	4	30
		♀	8	2	5	
7	FA	♂	12	3	0	3·3
		♀	11	3	1	
8	EAC	♂	12	3	0	0
		♀	11	4	0	
1‡	Nil	♂†	11	3	0	**0**
		♀	12	3	0	
4‡	FA	♂*†	11	2	0	**3·6**
		♀	12	2	1	

* One mouse accidentally killed.
† Tumour unsuitable for investigation in one mouse.
+ For details of scale see text.
‡ For details see part b.

Discussion

It was decided to ignore + haemolysis as the time that elapsed between the death of the mouse and the centrifugation of the tumour ascites could not be determined accurately ; differences of up to 18 hr. might have occurred. It was thought, however, that gross changes, i.e. ++ haemolysis, were unlikely to have been brought about in this way—and were as likely to occur in all groups concerned on this basis.

It was found that this degree of haemolysis was virtually confined to mice in group 6, that is to say to the mice that had been given tumour plus adjuvant subcutaneously. The tumour used for injection consisted of whole ascitic fluid. No attempt was made to remove the erythrocytes, which gave a PCV of 1 per cent Wintrobe, as such a procedure might have been detrimental to the tumour cells. Therefore the tumour adjuvant mixture also contained erythrocytes.

The results of the experiment suggest that the combination of erythrocyte-adjuvant may have been the cause of the increased haemolysis in the experimental group. It did not occur following the administration of whole ascitic fluid alone, i.e. tumour, erythrocytes and ascitic serum (group 8), and there was only a very

minor non-specific reaction following subcutaneous adjuvant alone. It was also absent in the untreated controls.

Considering these findings in relation to those in the main experiment (Hartveit, 1962) it could be argued that it was this haemolysis that was detrimental to the mice in the experimental group, and that it was this, and not a reaction against the tumour protein, that played a major part in their early death. If only the survival time is considered this appears quite possible, but when taken in conjunction with the tumour blood content it is evident that this is unlikely. While one could well expect treatment with an erythrocyte-adjuvant mixture to increase the haemolysis of the blood present in the tumour, one could hardly expect that to account for an increase in the blood content itself.

Secondly it could be argued that group 6 showed the greatest amount of haemolysis as the tumours contained the greatest amounts of blood. However, on referring back to Table III of the original paper (Hartveit, 1962) it will be seen that the females in group 7 showed almost as high a tumour blood content as those in group 6, while they do not show a correspondingly high degree of haemolysis.

Thus the haemolysis will have detracted from the findings on the blood content of the tumour ascites in group 6 as judged from the packed cell volume of the erythrocytes present. The amount of blood present was, in fact, even greater than that recorded in this group. It is of note that this reaction to a normal tissue did not show a sex difference while that related to the tumour tissue did.

SUMMARY

(a) Emulsification of Ehrlich's ascites carcinoma with Freund's adjuvant does not appear to have adversely effected the growth of the tumour cells in the mixture when this is injected subcutaneously into healthy mice. On subcutaneous injection of the tumour the female mice show greater natural immunity than the males as evidenced by their longer survival time.

(b) A non-specific immune type reaction to Ehrlich's ascites carcinoma was found in male mice following the subcutaneous injection of Freund's adjuvant mixture 30 days before the intraperitoneal injection of Ehrlich's ascites carcinoma. Female mice showed no reaction after this time interval, and the time interval had no effect on the reaction at the site of the subcutaneous adjuvant injection. Further the immune reaction was not dependent on the latter. While the mechanism of the immune reaction is thought to be similar to that occurring in female mice when the adjuvant is given on the same day as the tumour, it is suggested that it differs from that accompanying natural immunity and from the immunity acquired after the subcutaneous injection of Freund's adjuvant plus living tumour.

(c) It was found that haemolysis occurred in intraperitoneal Ehrlich ascites carcinoma following the subcutaneous injection of whole Ehrlich ascites carcinoma plus Freund's adjuvant. Marked haemolysis was absent in the untreated control groups. This haemolysis is thought to have been due to the erythrocytes present in the ascitic tumour fluid that was combined with the adjuvant mixture. The haemolysis will have detracted from the findings in the main experiment; the haemorrhagic response to the intraperitoneal tumour following the subcutaneous

injection of tumour plus adjuvant being in fact greater than that given by the PCV of the erythrocytes.

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Made and printed in Great Britain for
H. K. Lewis & Co. Ltd., by
Adlard & Son, Ltd., Bartholomew Press, Dorking.

Reprinted from
The British Journal of Cancer,
1962, Vol. XVI, p. 556

CELLULAR INJURY IN UNTREATED EHRLICH'S ASCITES CARCINOMA

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Received for publication May 28, 1962

It has previously been reported (Hartveit, 1961*b*) that the blood content of the tumour ascites produced by the mice used at this Institute after the intraperitoneal injection of Ehrlich's ascites carcinoma is a measure of their immune reaction to the homografted tumour. When this reaction is strong it kills the mice. Thus their survival time cannot be used to measure the possible injury to the tumour cells that would be expected on the basis of a specific homograft reaction.

It was therefore decided to investigate the tumour for evidence of cellular injury. An experimental group was set up in which the tumour dose was such that the first mice could be expected to die on the seventh day after the injection. All the mice were killed on the sixth day. Wet preparations of the tumour ascites were examined for non-viable cells and films were examined for cytological changes. The cytological changes following non-specific autolysis *in vitro* were also studied and the changes compared to those seen *in vivo*.

MATERIAL AND METHODS

The mice and the Ehrlich ascites carcinoma used were similar to those in previous experiments (Hartveit, 1961*b*), the tumour now being in its 124th transplant generation.

Experimental procedure.—One mouse provided the tumour for the experimental group which consisted of 15 male and 15 female mice. These mice were each given 0·1 ml. of the tumour ascites intraperitoneally (tumour cell count 2,740,000/c.mm., tumour blood content 0·2 per cent). To investigate the changes on non-specific autolysis the remaining tumour from the donor mouse was collected in a test-tube and left at 20° C., films and vital cell counts being made at intervals.

The mice in the experimental group were all killed 6 days later. Vital cell counts were carried out and films were made immediately from the tumour ascites, 1 ml. of which was centrifuged to obtain the percentage blood content (Hartveit, 1961*a*).

Vital staining and vital cell counts.—Schrek's method (1936) was used. The number of stained cells/500 unstained cells was expressed as a percentage.

Films.—These were air dried and stained at once with Leishman's stain.

Cell measurements and cell counts.—An eyepiece with a calibrated scale was used. Measurements were taken in the axis of the cell parallel to the scale, consecutive mononuclear cells being measured. Cells impinging on the scale were counted.

Donor mouse.—The nuclear and cell diameters of the tumour cells were measured in the immediate preparation and as they passed through the different stages of autolysis. The average of 100 readings was taken.

Experimental group.—The number of abnormally staining tumour cells (*vide infra*)/500 normal tumour cells was counted and expressed as a percentage.

RESULTS

Experimental group

Films.—In normal Ehrlich ascites carcinoma cells stained with Leishman's stain the nucleus is a dark bluish purple, the nucleoli even darker and the cyto-

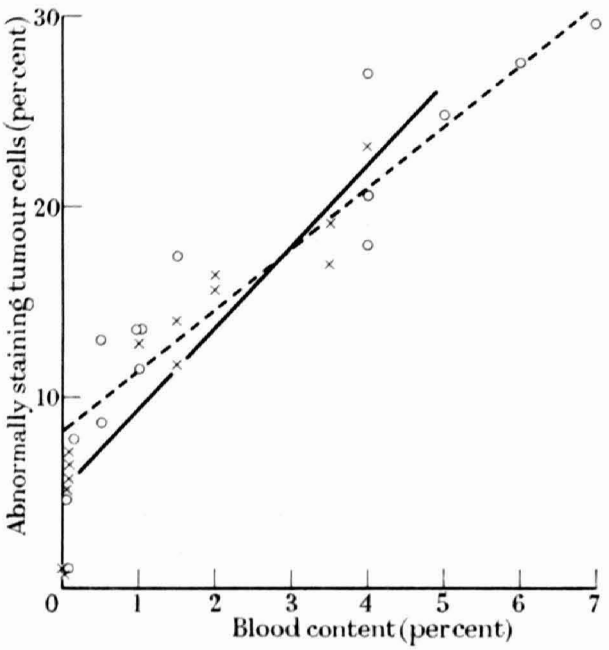


FIG. 1.—Scatter diagram and regression lines for the number of abnormally staining tumour cells in the tumour ascites related to its blood content.

1. Male values ———— and ×.
2. Female values — — — and ○.

Note. Tumour unsuitable for investigation in one male mouse.

plasm dark blue. In these animals some of the tumour cells stained differently; the nucleus being reddish purple to red, the nucleoli light blue and the cytoplasm slate grey.

Fig. 1 shows the scatter diagrams and regression lines for the number of abnormally staining tumour cells (y) in the immediate preparation related to the blood content of the tumour ascites (x) in both sexes. (Male $y = 5{\cdot}02 + 4{\cdot}27x$ and female $y = 8{\cdot}25 + 3{\cdot}17x$.) The difference in the numbers of these cells and in the tumour blood content between the sexes is not statistically significant. The correlation coefficient (r) for the relationship between these two factors is

0·91 for the males and 0·87 for the females. This positive correlation is significant in both sexes ($0{\cdot}01 > P > 0{\cdot}001$).

Table I gives the mean cell and nuclear diameters of these cells and of the normal tumour cells in air dried preparations. The abnormally staining cells are considerably larger. The difference in both diameters is significant ($0{\cdot}001 > P$).

TABLE I.—*The Mean Cell Diameter (SD) and the Mean Nuclear Diameter (SD) of the Normal and of the Abnormally Staining Tumour Cells (Air Dried Preparation)*

Ehrlich ascites carcinoma cells	Diameter, μ			
	Cell	SD	Nucleus	SD
Normal . . .	13·86	1·65	10·75	1·32
Abnormally staining .	21·00	3·80	15·80	2·64

The morphology of these cells also differed from that of the normal tumour cells. The surface of the cell was covered with cytoplasmic blebs, the cytoplasm was finely granulated and often contained more vacuoles than normal. The large nucleus contained a network of nuclear protein with clear nuclear sap between and the nucleoli were prominent. Fig. 2 shows one of these cells with some normal tumour cells for comparison.

Some of the abnormally staining cells appeared to be disintegrating. Rupture of the cell membrane with loss of cytoplasm preceded nuclear changes. The formation of nuclear blebs preceded loss of nucleoli (Fig. 3). Increase in the size of these blebs resulted in the rupture of the nuclear membrane and loss of nucleoli. Clumps of free nuclear material were also seen.

Vital staining.—Neither the normal tumour cells nor the cells with cytoplasmic blebs took up the stain after 2 minutes. After 10–15 minutes a few of the cells with blebs did so.

Tumour from donor mouse

The immediate preparation contained 0·8 per cent of the large abnormally staining tumour cells.

On autolysis the normal tumour cells showed a series of morphological changes similar to those described by King, Paulson, Puckett and Krebs (1959) following irradiation and salyrgan. These changes consisted of cytoplasmic granulation, the formation of coarse blebs on the cell surface, swelling of the cytoplasm and of the nucleus and were accompanied by changes in staining reaction similar to those in the large abnormally staining cells in the immediate preparation (Fig. 4). This was followed by rupture of the cell, pyknotic condensation, reswelling and fatty degeneration of the denatured protein with the formation of ghost cells.

Table II shows the mean nuclear diameter of the tumour cells at the different stages of autolysis compared to that of the healthy tumour cells and the large abnormally staining cells. The autolytic tumour cells never reached the size of the abnormally staining cells. The difference in nuclear diameter between the largest autolytic cells and the latter cells is significant ($0{\cdot}001 > P$).

Vital staining.—Table III shows that the number of stained cells increased with time and that the increase was not strictly parallel to the increase in the number of abnormally staining autolytic cells seen on the films.

TABLE II.—*The Mean Nuclear Diameters (SD) of the Tumour Cells on Autolysis compared to that of the Large Abnormally Staining Tumour Cells and of the Normal Tumour Cells (Air Dried Preparation)*

Type of Ehrlich ascites carcinoma cell	Nuclear diameter (μ)	SD
Early autolytic (minimum—4 hours)	12·73	1·54
Early autolytic (maximum—168 hours)	13·92	1·85
Pyknotic	6·80	0·94
Ghost	12·44	1·53
Large abnormally staining	15·80	2·64
Normal	10·75	1·32

TABLE III.—*Percentage of Vitally Stained Cells and of Abnormally Staining Cells in Autolytic Tumour Related to Time*

Time (hours)	Vitally stained cells (%)	Abnormally staining autolytic cells (%)
0	0	0·0
3	0	—
4	3	20·0
48	92	49·2
72	100	56·6

DISCUSSION

As Ehrlich's ascites carcinoma is a homograft one would expect to find evidence of injury to the cells following transplantation. Vital staining by Schrek's method (1936) is used extensively as evidence of cell death (Parker, 1961). However the assumption that all the cells taking up the stain are dead has been questioned (British Empire Cancer Campaign, Annual Report, 1951), and the conclusion reached was that while vital staining cannot be taken as certain evidence of cell death, all cells that do not take up the stain are viable.

With regard to Ehrlich's ascites carcinoma it has been shown that vital staining occurs just before the cell bursts and becomes pyknotic (King, Paulson, Puckett and Krebs, 1959). Cytological changes preceding vital staining were demonstrated in the autolytic tumour in the present work (Table III). These changes are thus an indication of cell injury but not of cell death.

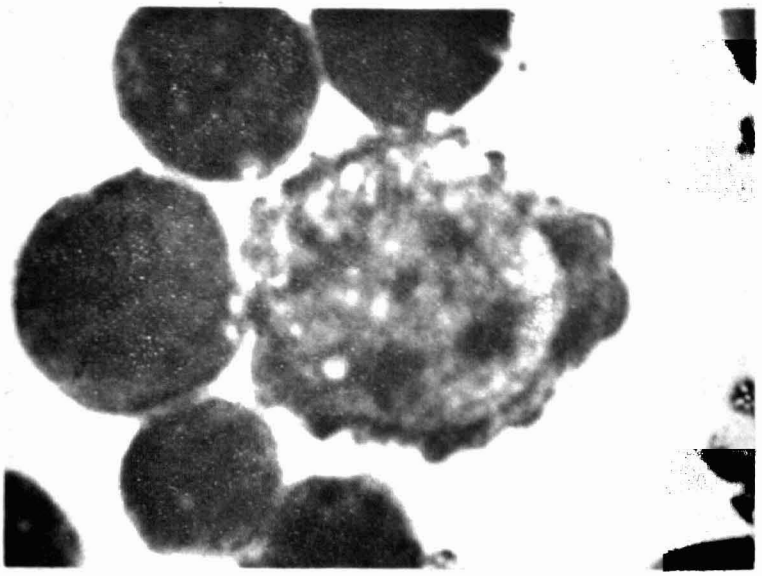
The large cells found in the tumour in the experimental group are then probably, on the basis of their staining reaction, injured cells. Some of them, after prolonged exposure, will take up the vital stain. These injured cells were larger than the normal cells, and larger than the autolytic cells ever became (Table II). Increase in the size of tumour cells following injury is a common finding (Lumsden,

EXPLANATION OF PLATE

FIG. 2.—Large abnormally staining tumour cell with cytoplasmic blebs. Note size in comparison to normal tumour cells. Leishman's stain ×650.

FIG. 3.—Large abnormally staining tumour cell in process of disintegration. Note nuclear bleb formation. Leishman's stain ×650.

FIG. 4.—Early autolytic changes in normal tumour cell. Note size in comparison to large abnormally staining cell in Fig. 2. Leishman's stain ×650.



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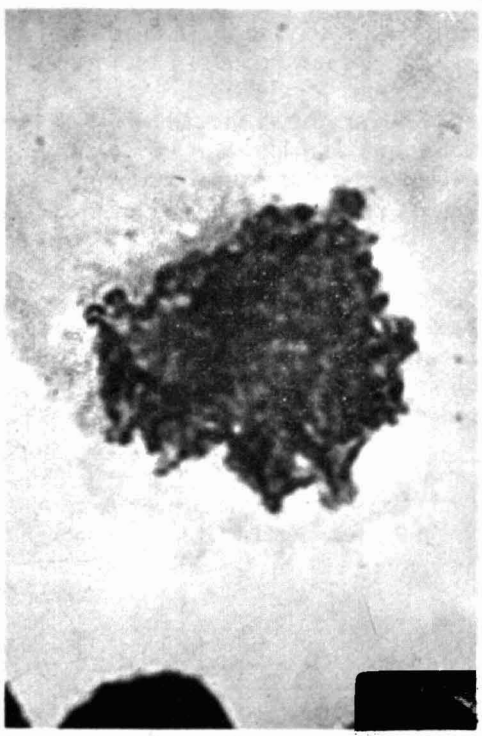

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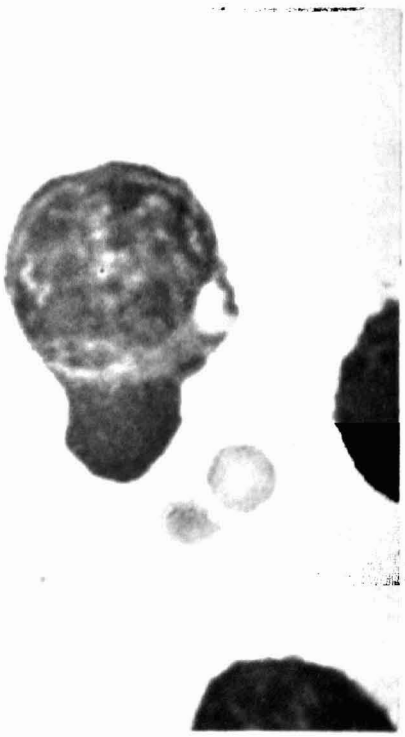

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1931 ; Kidd, 1953 ; Klein and Forssberg, 1954 ; Flax, 1956 ; Green, Barrow and Goldberg, 1959) and it appears that the degree of swelling may vary with the different forms of injury (Koller and Casarini, 1952 ; Kalfayan and Kidd, 1953 ; Ahlström and Ising, 1955 ; King, Paulson, Puckett and Krebs, 1959).

Of the many cytological changes described following injury the changes in the large cells in the present experimental group were most like those described by Flax (1956) and Green, Barrow and Goldberg (1959) following treatment with a specific antitumour serum from a foreign host. Flax saw cytoplasmic changes and rupture of the cell membrane in air dried preparations, but he considered them to be artefacts. Green, Barrow and Goldberg describe similar cytoplasmic changes seen on phase contrast and electron microscopy. They did not see rupture of the cell membrane. In the present work the cytoplasmic changes were seen in wet as well as air dried preparations and the rupture of the cell membrane was seen to represent a stage in the disintegration of the cell.

The striking difference between the cells described by Flax and those reported here is that his cells formed ghost cells in the manner of the autolytic cells in this material. According to Green, Barrow and Goldberg more cytoplasmic swelling occurs in a low protein medium. As the protein content was probably much higher in Flax's experiment and in the autolytic tumour, in both of which a high proportion of the cells were degenerating, than in the tumours in this experimental group, the difference in medium could explain this discrepancy in the findings. Flax's findings also indicate that the formation of ghost cells is not related to the temperature of the medium.

The ghost cells in Flax's experiment were not preceded by pyknotic cells as was the case with the autolytic cells. It may be that in the autolytic cells degradation of the cytoplasmic and nuclear protein proceeded at the same rate while in his cells the cytoplasmic protein was denatured first and modified the disintegration of the nucleus. The relative lack of cytoplasmic protein in the large cells in the present experiment could account for the lack of modification in the nuclear degeneration.

Thus these large cells show changes characteristic of injury by specific antitumour antibody. The sequence of events suggests that the noxious agent acted on the surface exposed to the medium and points to the presence of a cytotoxic factor in the ascitic fluid ; this possibility is being investigated further.

The present work thus indicates that cellular injury does occur following the homotransplantation of Ehrlich's ascites carcinoma. It also shows that the number of cells injured varies from mouse to mouse (Fig. 1) as is to be expected as the mice used were heterozygous. The high positive correlation between the blood content of the tumour and the cellular damage supports the idea that they are both consequences of the same basic response, an immunity or homograft reaction.

SUMMARY

The presence of injured cells in untreated Ehrlich ascites carcinoma is reported. The cytological changes were found to give a more delicate indication of cellular injury than vital staining. The morphological characteristics of the injured cells were similar to those described following treatment with specific antitumour serum, and indicate that the cells were damaged through a specific immune (homograft) reaction on the part of the host. This view is supported by the finding

of a high positive correlation between the number of these cells present and the blood content of the tumour ascites, which is a measure of such a reaction (Hartveit, 1961*b*).

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Since this paper went to press a further paper (Lindner, 1960) has come to the notice of the author. The morphological changes described in Ehrlich ascites carcinoma cells, following treatment with specific immune gamma globulin and supported by fluorescent antibody studies, appear identical to those described in untreated tumour in the present paper. This further supports the idea that these changes are the result of an immune reaction of the mice to the homografted tumour.

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Made and printed in Great Britain for
H. K. Lewis & Co. Ltd., by
Adlard & Son, Ltd., Bartholomew Press, Dorking.

Separatum
ACTA PATHOLOGICA ET MICROBIOLOGICA SCANDINAVICA
Vol. 58. Fasc. 1. 1963

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THE IN VITRO DEMONSTRATION OF A CYTOTOXIC FACTOR IN EHRLICH'S ASCITES CARCINOMA

By

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Received 14.viii.62

In a previous experiment (*Hartveit* 1962) the author found that the number of injured cells present in untreated Ehrlich ascites carcinoma at 6 days increases with the blood content of the tumour. When the blood content is particularly high the number is less than would be expected from the regression line. This finding, which has not been stressed previously, prompted the re-examination of the films from the tumours in the previous experiment. It was found that in the cases in which the number of injured cells was lower than expected many pyknotic cells were also present, while there were few in tumours with a low blood content.

In view of these findings, which suggest that there is a factor injurious to the tumour cells—a cytotoxic factor—in the ascitic fluid, it was decided to see if an excess of fluid over that present in vivo would increase the amount of cell damage produced (experiment I), and if this were so to see whether the different fluids varied in their ability to cause cell damage, and whether this ability was related to the tumour blood content (experiment II).

MATERIAL AND METHODS

The mice and the Ehrlich ascites carcinoma used were similar to those used in previous experiments (Hartveit 1961), the tumour now being in its 132nd. transplant generation.

Experimental Procedure.

Experiment I. One mouse provided the tumour for the experimental group which consisted of 5 male and 5 female animals that were each given 0.1 ml of the tumour ascites intraperitoneally (tumour cell count 1,370,000/mm. c., tumour blood content —2 per cent). After 6 days the mice were killed and the tumour ascites removed. Half of the tumour ascites from each mouse was centrifuged to obtain the cell-free ascitic fluid. This supernatant was then added to the other half of the tumour ascites, using Pasteur pipettes, in the proportions shown in Table 1. Films were made, as described previously (Hartveit 1962), from the original tumours and from all dilutions.

Experiment II. (Based on the results of experiment I, vide infra). One mouse provided the tumour for this experimental group which consisted of 15 male and 15

female animals. These were each given 0.1 ml of the tumour ascites intraperitoneally (tumour cell count 1,960,000/mm. c., tumour blood content—3 per cent).

Six days after the injection of the tumour all the mice were killed. One ml of the tumour ascites was centrifuged in a Wintrobe tube to obtain the PCV of the erythrocytes (*Hartveit* 1961) and the cell-free supernatant ascitic fluid. This supernatant was then added to the original tumour as in the above experiment and films were made in the same way. The films were numbered 1–5 according to the amount of excess ascitic fluid used (Table 1). The end point of the reaction was recorded as the number of the tube in which all the tumour cells were either swollen or pyknotic, *i.e.* at which no healthy cells remained.

TABLE 1

The Proportions of Whole Ehrlich Ascites Carcinoma in Cell-Free Ascitic Fluid from the Same Mouse Used in the in vitro Tests.

Tube number	Whole tumour, drops	Ascitic fluid, drops
1	1	0
2	2	1
3	1	1
4	1	2
5	1	4

RESULTS

Experiment I. Figure 1 shows the result of adding different amounts of ascitic fluid from the same tumour to the tumour cells (1a). The addition of small amounts of excess fluid resulted in the production of large injured cells (1b), greater amounts produced pyknosis (1c) and still greater amounts clumping (1d). The pyknotic cells were seen to be joined by fine bridges (Fig. 2a). These bridges were also seen on dark ground illumination of the wet preparation (2b). These findings were constant in all the mice investigated.

TABLE 2

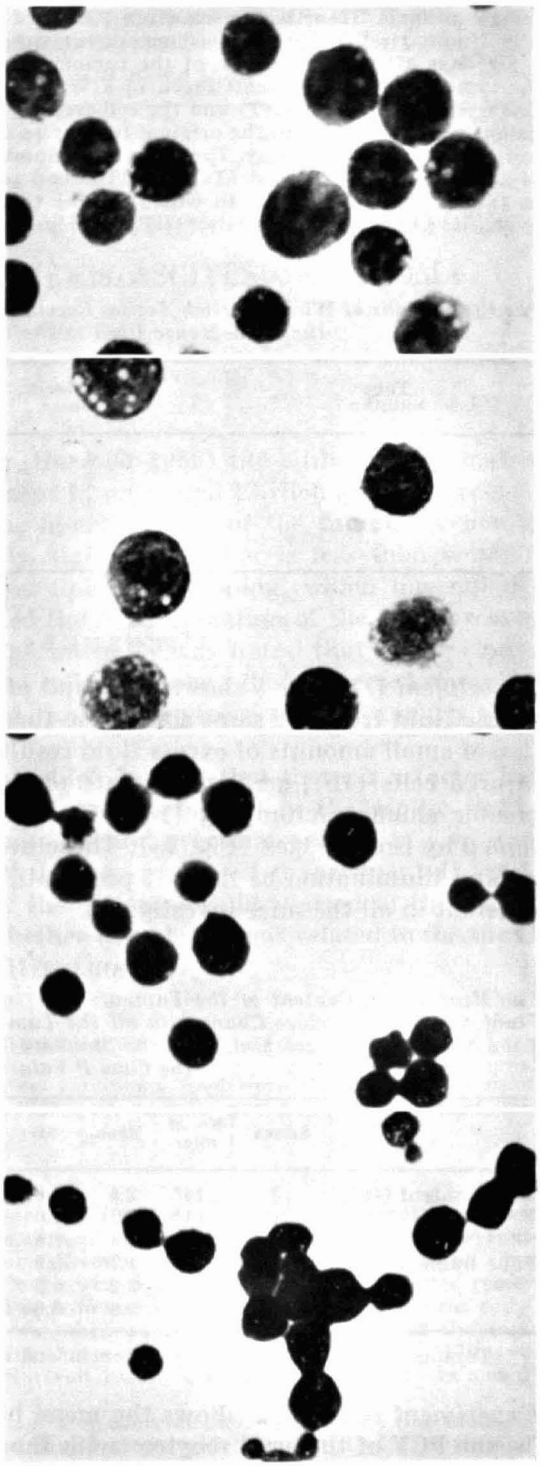
The Mean Blood Content of the Tumours (SD) and the Amount of Excess Ascitic Fluid Needed to Produce Changes in all the Tumour Cells—Expressed as the Mean Tube Number (SD), see text, with the Standard Error (SE) of the Sex Difference, the t and P Values.

	Series	No. of mice	Mean	SD	SE	t	P
Blood content (%)	♂	14*	2.5	1.9	0.88	1.46	$0.2 > P > 0.1$
	♀	14*	1.2	1.6			
	♂ + ♀	28	1.8	1.9			
Tube number	♂	14*	1.7	0.9	0.33	1.52	$0.2 > P > 0.1$
	♀	14*	2.2	0.9			
	♂ + ♀	28	2.0	0.9			

* Tumour unsuitable for investigation in one mouse.

Experiment II. Table 2 shows the mean blood content of the tumours, *i.e.* the PCV of the erythrocytes (with the standard deviation, SD) and

Fig. 1.
The effect of an excess of ascitic fluid from the same tumour on Ehrlich ascites carcinoma cells. Leishman's stain, × 720, (reduced to approx. × 580 in print).

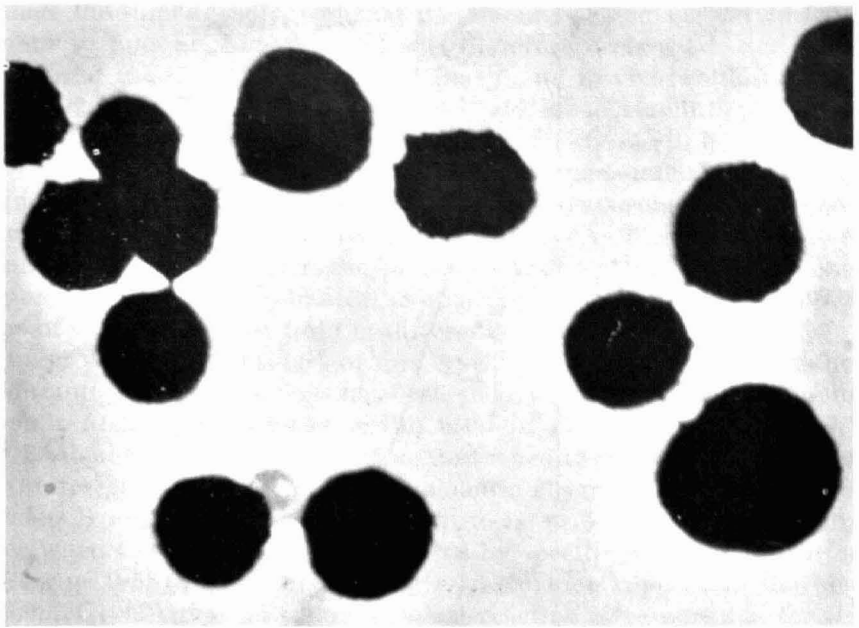


a. Whole tumour. Note normal cell size.

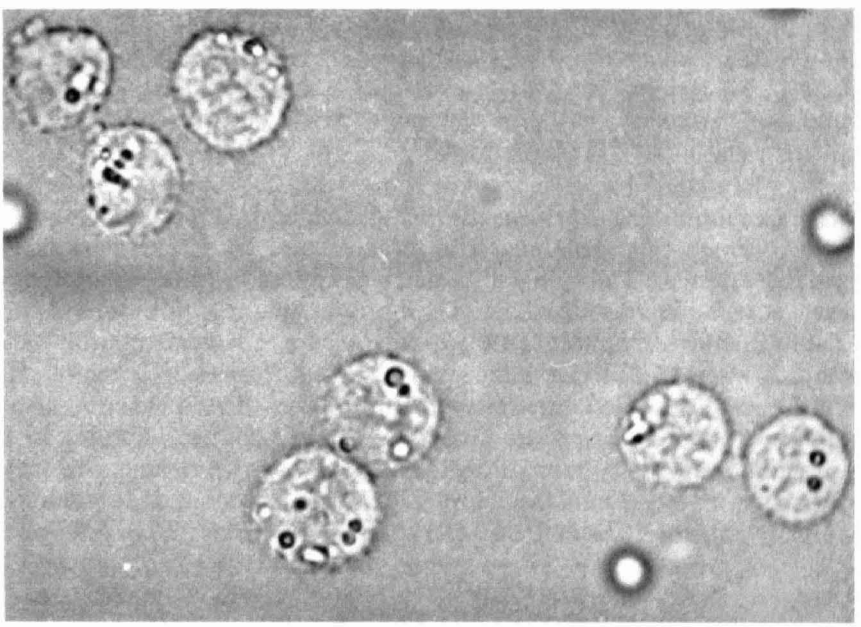
b. 1 drop of tumour plus 1 drop of ascitic fluid. Note swollen cells and loss of cytoplasm.

c. 1 drop of tumour plus 2 drops of ascitic fluid. Note pyknosis.

d. 1 drop of tumour plus 4 drops of ascitic fluid. Note pyknosis and clumping.



a. Leishman's stain, × 1400.



b. Unstained wet preparation, dark ground illumination, × 1100.

Fig. 2.

Bridge formation between pyknotic Ehrlich ascites carcinoma cells following the addition of an excess of cell-free ascitic fluid from the same tumour to the unwashed cells.

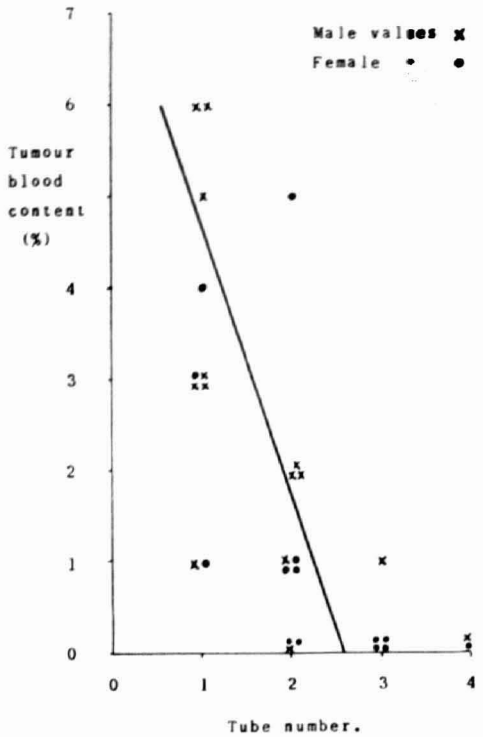


Fig. 3.

Regression line and scatter diagram of the relationship between the tumour blood content and the amount of excess tumour ascitic fluid needed to injure all the tumour cells—expressed as the tube number (see text), for the total series.

the mean amount of excess ascitic fluid needed to produce changes in all the tumour cells (SD) for the male, female and total series. The standard error (SE) of the difference between the male and female means is also given, with the t and P values.

Table 2 shows that the sex difference was not significant for either factor. Therefore the total series was used for the regression line (Fig. 3) of the relationship between the tumour blood content (x) and the amount of excess ascitic fluid needed to produce changes in all the tumour cells (y), ($y = 2.58 - 0.33x$). The corresponding correlation coefficient, r, was found to be –0.6934 ($t = 3.602$) and as such was highly significant ($0.01 > P > 0.001$). ($r♂ = -0.6766$, $r♀ = -0.6517$).

DISCUSSION

The hypothesis on which the present experiments are based is that there is a cytotoxic factor in the ascitic fluid surrounding the Ehrlich ascites carcinoma cells. A previous experiment (*Hartveit* 1962) had suggested that in some cases enough of this factor is present in vivo to

damage the tumour cells, and that the amount of damage varied from tumour to tumour. Experiment I was therefore designed to see if the addition of ascitic fluid in excess of that found in vivo would increase the cell damage. The results show that it did, small amounts producing the type of injury, seen in vivo, that has been shown to be related to the tumour blood content (*Hartveit* 1962); larger amounts producing pyknosis and clumping of the tumour cells. The formation of bridges between the pyknotic cells preceded clumping. As these bridges were seen in the wet preparations they are not fixation artefacts. The phenomenon is not purely mechanical as it occurred with different proportions of cells and ascitic fluid in different tumours (vide infra).

Direct reference to bridges of this type between tumour cells has not been found, but such a bridge has been clearly demonstrated in a photograph in *Fitch's* work (1962) on this tumour. This photograph (*Fitch's* Fig. 2A) shows the result of treating unwashed tumour cells with specific fluorescent labelled anti-tumour globulin absorbed with mouse liver powder. Bridges have also been shown to be present between erythrocytes when they have been agglutinated by specific antibody (*Stratton & Renton* 1958). Thus this finding adds further support to the idea (*Hartveit* 1962) that an immunological reaction is responsible for the cell damage.

Experiment II was designed to see if the amount of this factor varies in the different ascitic fluids, and whether it is related to the tumour blood content, as suggested from the findings in a previous experiment (*Hartveit* 1962). It was found that the different fluids did vary (Fig. 3) in their ability to injure the cells of the tumour from which they were taken. It was also clearly shown that this ability is related to the tumour blood content—a fluid from a tumour with a high blood content being more active than one from a non-haemorrhagic tumour. The considerable scatter in the results in Figure 3 may well be due to the experimental error inherent in the use of Pasteur pipettes, as the drop size was not uniform, different pipettes being used for tumour and ascitic fluid.

The results of these experiments provide further evidence of the existence of a cytotoxic factor in untreated Ehrlich ascites carcinoma, and suggest that this factor is related to the immune reaction to the homografted tumour that is reflected in the tumour blood content (*Hartveit* 1961). There are two possible explanations of the findings. The cytotoxic factor may be present in the ascitic fluid that is added to the whole tumour. In this case an excess of the factor in relation to the cells is usually needed. The second possibility is that the factor is present on the unwashed cells and that an additional factor, present in the ascitic fluid, is needed in excess to trigger off the reaction. In this connection it is of note that the interaction of Ehrlich ascites carcinoma cells and immune antiserum from a foreign host has been shown to take place only when complement has been added to the sy-

stem (*Flax* 1956, *Wissler & Flax* 1957, *Fitch* 1962, *Stone, Dzoga & Wissler* 1962). Similarly, with the Yoshida ascites sarcoma, complement was needed to produce the in vitro changes in the tumour cells (*Lund* 1957). Further experiments on the mode of action of the cytotoxic factor in Ehrlich's ascites carcinoma are in progress.

SUMMARY

The presence of a factor in untreated Ehrlich ascites carcinoma that is capable of damaging the tumour cells in vitro is demonstrated. The morphology of the injured cells, and the formation of bridges between the pyknotic cells, suggest that an immunological reaction is involved. This is also in accordance with the finding that the activity of the tumours in this respect is proportional to their blood content, a measure of the immune reaction of the host to the tumour homograft (*Hartveit* 1961).

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Separatum
ACTA PATHOLOGICA ET MICROBIOLOGICA SCANDINAVICA
Vol. 58. Fasc. 1. 1963

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THE VIABILITY OF PYKNOTIC EHRLICH ASCITES CARCINOMA CELLS

By

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Received 14.viii.62

In describing the stages in the death of an Ehrlich ascites carcinoma cell *King, Paulson, Puckett & Krebs* (1959) have shown that the cell bursts before it becomes pyknotic. The findings in autolytic tumour support this view (*Hartveit* 1962b). The author has reported previously (*Hartveit* 1963) that Ehrlich ascites carcinoma cells treated with an excess of ascitic fluid from the same tumour become swollen, if the amount of excess fluid is low, and pyknotic if the amount is greater. The latter cells are obviously damaged, but may or may not be dead. To settle this point the survival time of mice after the intraperitoneal injection of undiluted tumour, tumour diluted in physiological saline and tumour diluted with cell-free tumour ascitic fluid was studied.

MATERIAL AND METHODS

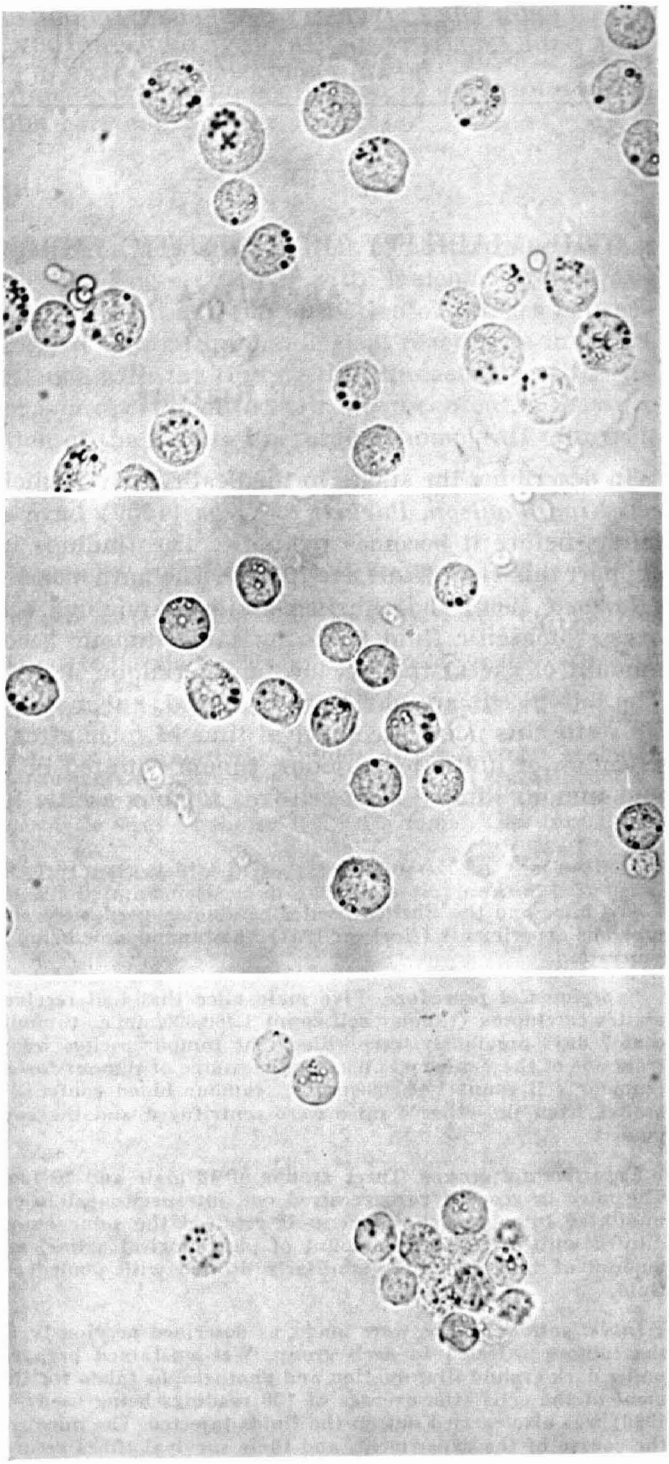
The mice and the Ehrlich ascites carcinoma used were similar to those used in previous experiments (*Hartveit* 1961), the tumour now being in its 130th. transplant generation.

Experimental procedure. Five male mice that had received 0.1 ml of Ehrlich's ascites carcinoma (tumour cell count 1,360,000/mm.c., tumour blood content—2 per cent)7 days previously were killed. The tumour ascites was removed. The tumour from one of these mice was used as the source of tumour for all experimental groups (tumour cell count 1,480,000/mm. c., tumour blood content—a trace). The tumour ascites from the other 4 mice were centrifuged and the supernatant ascitic fluid pooled.

Experimental groups. Three groups of 10 male and 10 female mice were set up. The mice in group I each received one intraperitoneal injection of 0.1 ml of the undiluted tumour ascites. Group II received the same amount of tumour ascites diluted with 5 times the amount of physiological saline, and group III the same amount of tumour ascites similiarly diluted with pooled cell-free tumour ascitic fluid.

Investigations. Films were made, as described previously (Hartveit 1962b), from the tumour injected in each group. Wet unstained preparations were examined under dark ground illumination and photographs taken for the subsequent measurement of the cells (the average of 100 readings being used). Vital staining (*Schrek* 1936) was also carried out on the fluids injected. The mice were weighed 5 times in the course of the experiment, and their survival times recorded.

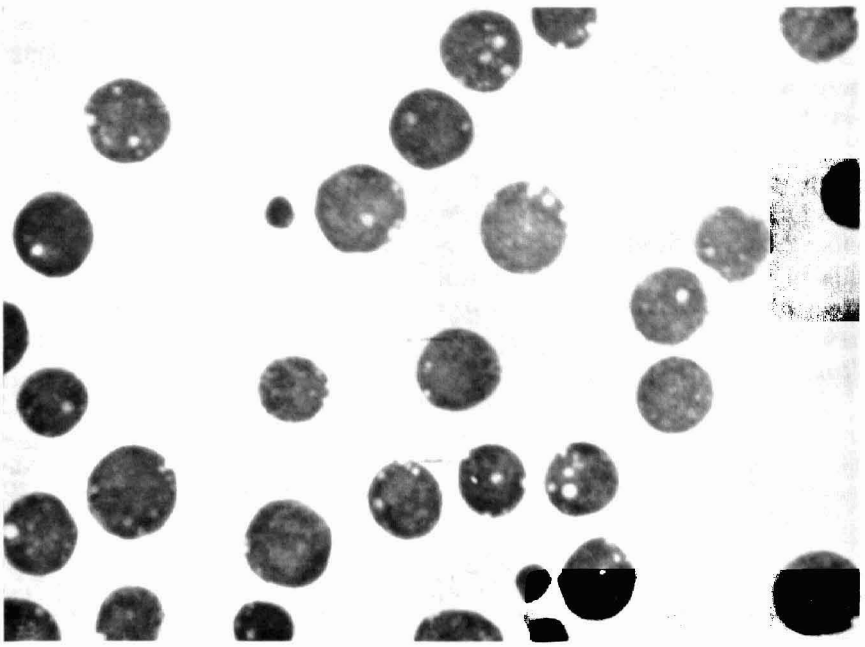
Fig. 1.
The Ehrlich ascites carcinoma cells injected in the 3 groups.
Dark ground illumination, × 560 (reduced to approx. × 450 in print).



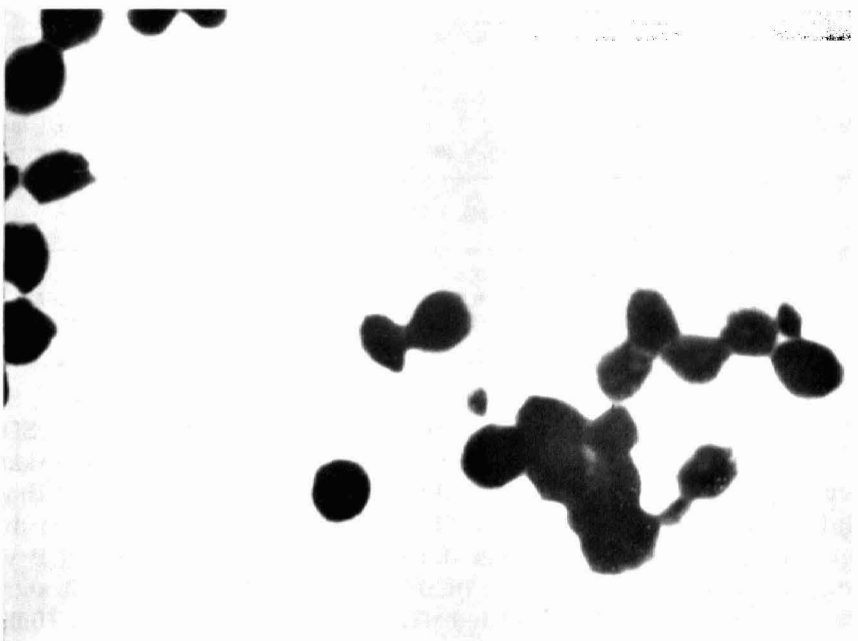
a. Group I. Note healthy cells.

b. Group II. Note slight shrinkage.

c. Group III. Note pyknosis and clumping.



a. Group I. Note normal cell size.



b. Group III. Note pyknosis and clumping.

Fig. 2.

The Ehrlich ascites carcinoma cells injected in group I, undiluted, and in group III, diluted with ascitic fluid. Leishman's stain, × 700.

RESULTS

Figure 1 shows wet preparations of the tumour injected in each of the experimental groups. The tumour cells in Figure 1a—group I, injected undiluted—appear healthy. Some of those in Figure 1b—group II, diluted with saline—are slightly shrunken. The cells in Figure 1c—group III, diluted with ascitic fluid—are pyknotic and show clumping. This pyknosis is further evident in Figure 2 which shows the undiluted cells (2a) and the cells diluted in ascitic fluid (2b). The pyknosis of the cells in the latter fluid was uniform, no healthy cells remaining. The effect of diluting with pooled ascitic fluids appeared identical to that previously seen on adding an excess of fluid from the same tumour (*Hartveit* 1963).

TABLE 1 a

The Diameter (x) of the Ehrlich Ascites Carcinoma Cells in the 3 Groups, I—Given Undiluted Ehrlich Ascites Carcinoma, II—Tumour Diluted with Saline, and III—Tumour Diluted with Ascitic Fluid, as Measured from Photographs.

Group	$\bar{x}$ (mm)	$SD_{\bar{x}}$
I	9.16	1.41
II	8.38	1.17
III	6.56	0.73

TABLE 1 b

The Difference in Cell Diameter (x) between the 3 Groups (see Table 1 a), with the SE, t and P Values.

Difference between groups	Diff. in $\bar{x}$ (mm)	SE	t	P
I and II	0.78	0.44	1.77	$0.1 > P > 0.05$
II and III	1.82	0.69	2.64	$0.02 > P > 0.01$
I and III	2.60	0.39	6.72	$0.001 > P$

Table 1a gives the mean cell diameter, with standard deviation (SD), of the cells injected in each group, and shows that slight shrinkage occurred in cells diluted with saline and marked shrinkage in those diluted with ascitic fluid. Table 1b shows the differences in cell diameter between the groups, with the standard error (SE), t and P values. The amount of shrinkage in the saline diluted cells is not significant, while that of those diluted with ascitic fluid is highly so ($0.001 > P$).

Table 2 gives the survival time (SD) of the mice in all three groups and the sex differences within the groups, with SE, t and P values. The sex difference was only significant in group III ($0.05 > P > 0.02$).

TABLE 2

The Survival Time (x) in Days (SD) of the Mice in the 3 Groups (see Table 1 a). The Sex Differences within the Groups with SE, t and P Values Are Given. (10 Male and 10 Female Mice in each Group).

Group	Series	$\bar{x}$	SD$\bar{x}$	Sex diff. $\bar{x}$	SE	t	P
I	♂	13.5	2.4	1.5	0.93	1.61	$0.2 > P > 0.1$
	♀	15.0	2.2				
II	♂	15.4	2.7	1.8	1.24	1.45	„
	♀	13.6	2.8				
III	♂	14.4	1.9	2.3	1.08	2.12	$0.05 > P > 0.02$
	♀	16.7	2.8				

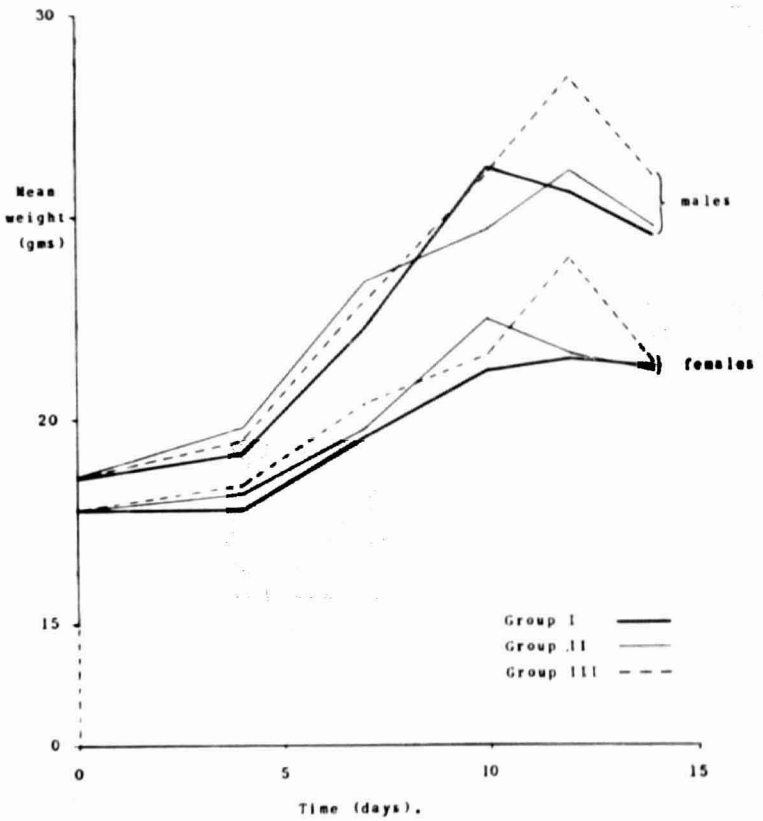


Fig. 3.

The mean weight of the mice in the different groups,

I – with undiluted Ehrlich's ascites carcinoma,
II – with tumour diluted with saline, and
III – tumour diluted with ascitic fluid, related to time after injection of the tumour.

Table 3 gives the difference in survival time between the groups, with SE, t and P values. The difference between the females in groups II and III was significant ($0.05 > P > 0.02$).

TABLE 3

The Difference in Survival Time (x) in Days between the 3 groups (see Table 1 a), with the SE, t and P Values. (10 Male and 10 Female Mice in each Group).

Difference between groups	Series	Sex diff. x̄	SE	t	P
I and II	♂	1.9	1.15	1.65	$0.2 > P > 0.1$
	♀	1.4	1.00	1.39	,,
II and III	♂	1.0	0.62	1.61	,,
	♀	3.1	1.26	2.47	*$0.05 > P > 0.02$*
I and III	♂	0.9	0.97	0.93	$0.4 > P > 0.3$
	♀	1.7	1.05	1.62	$0.2 > P > 0.1$

On vital staining of the tumour cells in the three fluids injected none of the tumour cells took up the vital stain at 2 minutes, at 15 minutes or at 45 minutes, at which time all the injections had been completed. After 2 hours 7 per cent of the cells in the group I fluid, 10 per cent in group II, and 50 per cent in the group III fluid took up the vital stain.

Figure 3 shows the weights of the mice in the 3 groups related to time. The differences between the groups are not statistically significant.

DISCUSSION

The results of this experiment show that although morphological signs of injury can be seen in Ehrlich ascites carcinoma cells treated with an excess of tumour ascitic fluid the cells are not dead. This is in keeping with the results of vital staining but not in keeping with the idea that the pyknotic tumour cells are cells that have burst. *King, Paulson, Puckett & Krebs* (1959) have shown that damaged tumour cells swell, burst and subsequently become pyknotic and take up the vital stain. The author found the same in cells undergoing non-specific autolysis (*Hartveit* 1962b). It has also been shown that when a small amount of excess ascitic fluid is added to the tumour cells swelling results, but when a large amount of fluid is added all the cells become pyknotic (*Hartveit* 1963), preliminary swelling has not been observed.

This suggests that the injury inflicted by a great excess of the fluid differs from the primarily cytoplasmic injury that follows a smaller amount of the same fluid, in that the cell does not burst. This idea is supported by the findings in the present experiment. Vital staining showed that the tumour cells that had been diluted with ascitic fluid

did not take up the stain after 2 minutes—as they should have done had they been burst cells (*King, Paulson, Puckett & Krebs* 1959). After 2 hours half of them did so—in contrast to the controls—confirming that some damage was present.

The survival time of the mice in the present experiment clearly indicates that the pyknotic tumour cells were not dead (Table 3—the difference in survival time between the untreated cells (group I) and those diluted with ascitic fluid (group III) was not significant. The weight curves for the mice in the different groups (Fig. 3) show that there was no preliminary lag in group III that could indicate that a lower dose of living cells had been compensated by an XYZ effect exerted by the dead cells present.

The changes in cell diameter and morphology (Figs. 1 and 2, and Table Ia) suggest that the cells in group III are damaged. This is supported by the results of the vital staining and of the survival time experiments in the females (vide infra). It has been shown previously that female mice have greater natural resistance to Ehrlich's ascites carcinoma than males (*Hartveit* 1962a). This does not usually show up on intraperitoneal injection. But in group III of this experiment the females survived significantly longer than the males ($0.05 > P > 0.02$), Table 2. This finding suggests that the cells were damaged so that they were more vulnerable than usual to the immune response of the host. This is further supported by the finding (Table 3) that the females in group III also survived significantly longer than those in group II ($0.05 > P > 0.02$). The group II cells had been diluted in saline, *i.e.* they had gone through the same mechanical process as those in group III, but their vitality was not impaired in contrast to that of the cells diluted in ascitic fluid.

It is of note that Ehrlich ascites carcinoma cells treated with heterologous immune gamma globulin show swelling. Whether or not rupture of the cells takes place is debated (*Flax* 1956, *Green, Barrow & Goldberg* 1959), but their viability is definitely reduced (*Lindner* 1960). The changes in such cells appear to be identical to those following a small amount of excess ascitic fluid, and the reduced viability is in keeping with the finding that the cells with the latter changes show vital staining after about 10 minutes (*Hartveit* 1962b), in contrast to the pyknotic cells in the present experiment that needed two hours.

It thus seems that the injury to the tumour cells following a great excess of ascitic fluid is reversible, at least in the early stages, while that following smaller amounts may not be. This possibility is being investigated.

SUMMARY

Ehrlich ascites carcinoma cells treated with an excess of tumour ascitic fluid become pyknotic. These pyknotic cells are not dead but injured. It is suggested that the pyknosis of the cells has not been pre-

ceded by cell rupture with consequent irreversible cell damage. The experiment also presents further evidence that female mice show greater natural immunity to the Ehrlich ascites carcinoma than male mice.

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Separatum
ACTA PATHOLOGICA ET MICROBIOLOGICA SCANDINAVICA
Vol. 58. Fasc. 1. 1963

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SEX DIFFERENCES IN THE INTRAPERITONEAL GROWTH OF EHRLICH'S ASCITES CARCINOMA

By

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Received 29.viii.62

Previous experiments have indicated that there is a difference in the reaction of male and female mice to the transplantation of Ehrlich's ascites carcinoma. This difference does not appear to effect the survival time of the animals following intraperitoneal injection of the tumour (*Hartveit* 1961a) but it does show up on subcutaneous injection (*Hartveit* 1962a). It does not seem to be reflected in the blood content of the intraperitoneal tumour (*Hartveit* 1961a & b) but it does show up on the transplantation of pyknotic tumour cells (*Hartveit* 1963b). From these experiments it appears that female mice may possess more natural immunity to the homotransplant than males.

The following experiment was designed to study the morphology of the Ehrlich ascites carcinoma cells at various times after transplantation to see if this would give any further information on the above mentioned sex difference.

MATERIAL AND METHODS

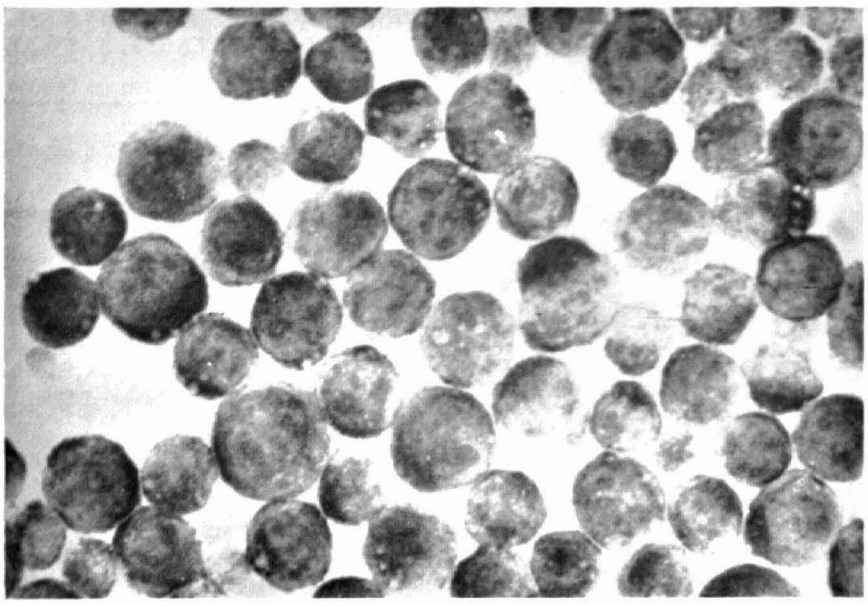
The mice and the Ehrlich ascites carcinoma used were similar to those used in previous experiments (*Hartveit* 1961 a), the tumour now being in its 133rd. transplant generation.

Experimental procedure. One male mouse, that had been injected with Ehrlich ascites carcinoma intraperitoneally 10 days previously, provided the tumour for the experimental group of 15 male and 15 female mice. Each of these was given 0.1 ml of tumour ascites intraperitoneally (tumour cell count 1,080,000/mm^3—tumour blood content—1 per cent).

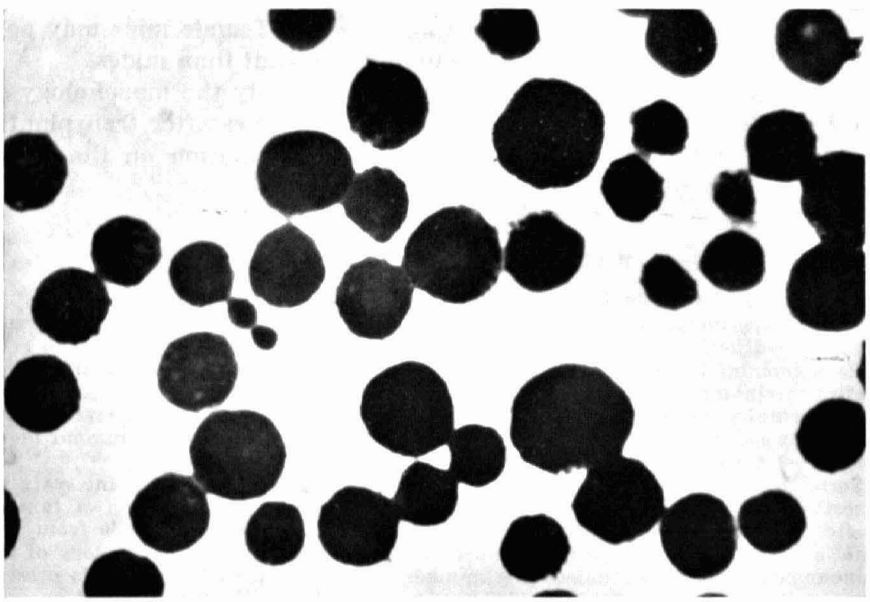
Serial biopsy specimens were taken from these mice at three-day-intervals by inserting a needle through the abdominal wall and collecting the drop of tumour ascites that formed when the needle was withdrawn. Films were made from this fluid and stained as described previously (*Hartveit* 1962 b). The morphology of the tumour cells was then studied. The number of large injured tumour cells present was counted as before (*Hartveit* 1962 b). Three hundred cells were counted on each film and the results expressed as a percentage.

RESULTS

Four main types of Ehrlich ascites carcinoma cells were seen. These were the normal cells (Fig. 1a), pyknotic cells (Fig. 1b) and two types



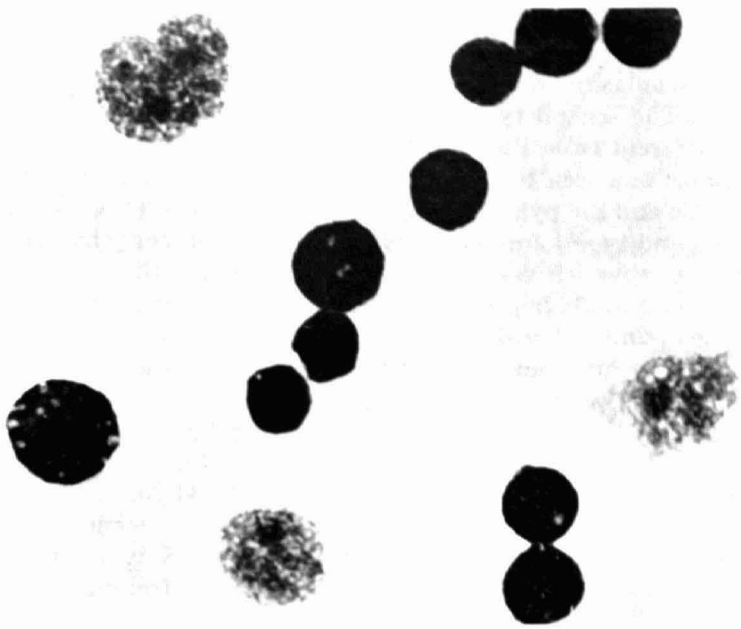
a. Biopsy specimen of tumour 3 days after tranplantation. Normal cells. Note size, shape and stainability.



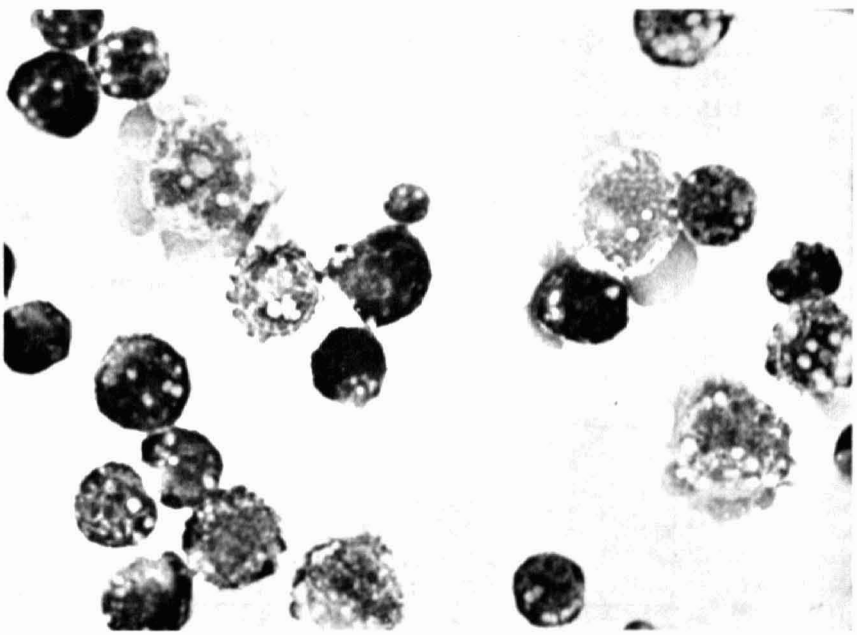
b. Biopsy specimen of tumour from the same mouse 9 days after transplantation. Note flattening of cell surface, pyknosis, increased stainability, intercellular bridges, and clumping.

Fig. 1.

Changes in untreated Ehrlich ascites carcinoma cells following transplantation. Leishman's stain, × 700.



a. Type 1, from first biopsy showing pyknosis. Note disintegration of cytoplasm and presence of nucleoli.



b. Type 2, from biopsy 9 days later. Note simultaneous disruption of cytoplasm and nucleus with loss of nucleoli. Also transition, via lesser changes of same type, from pyknotic to large cells.

Fig. 2.

Large injured tumour cells in untreated Ehrlich ascites carcinoma.
Leishman's stain, × 700.

of large injured tumour cell. The first of these (type 1) was similar to those described previously (*Hartveit* 1962b) that showed swelling and primary cytoplasmic damage indicative of immunological type damage (Fig. 2a). The second type of large injured tumour cell (type 2) was clearly different from the type 1 cells in that the nuclear and cytoplasmic damage was seen to occur simultaneously and transition stages between these and the pyknotic tumour cells were seen (Fig. 2b).

Normal and type 1 tumour cells were present in early biopsies. After a certain time, which varied from mouse to mouse, the majority of the tumour cells lost their previous spherical shape—their surface showed flat planes—and pyknosis occurred, with increased stainability. Intercellular bridges were also seen between these pyknotic cells and clumping was also present (Figs. 1a and 1b). A few type 1 cells were still present in the first biopsy showing pyknosis but after this time the tumour was made up of pyknotic and type 2 cells.

Figure 3 shows the time of occurrence of pyknosis. There was a marked sex difference. Pyknosis tended to occur earlier in the tumours in female mice. It was present in 6 of these at 3 days while none of the males had pyknotic tumours at this time. This difference is statistically significant ($0.001 > P$).

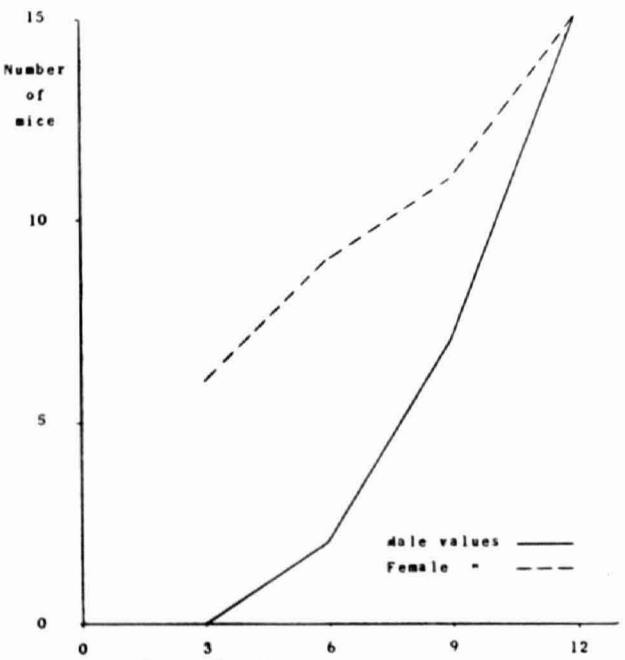


Fig. 3.
The time of occurrence of *in vivo* pyknosis in Ehrlich's ascites carcinoma. (15 ♂ and 15 ♀ mice).

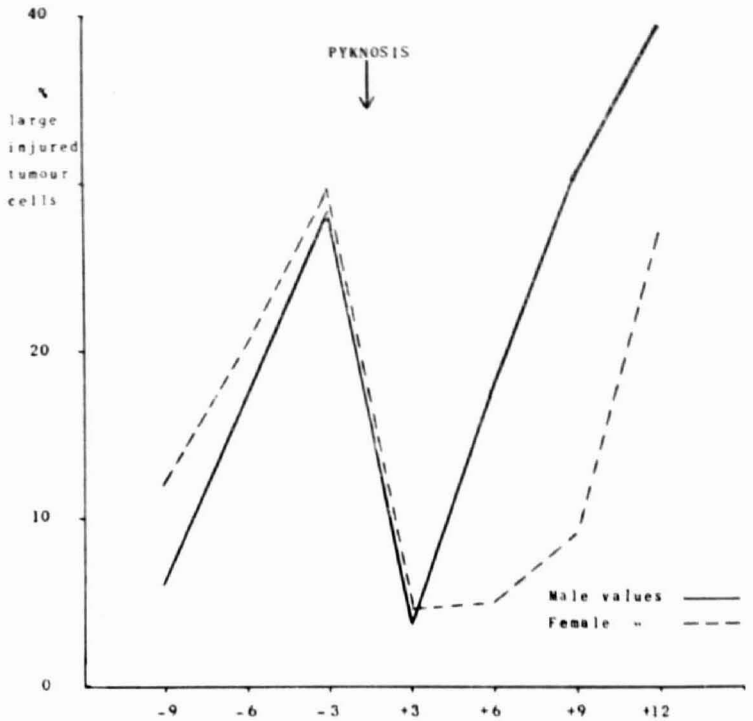


Fig. 4.

The mean percentage of large injured Ehrlich ascites carcinoma cells present in biopsies taken before and after *in vivo* pyknosis of the tumour (see Table 1).

TABLE 1

The Percentage of Large Injured Tumour Cells (Types 1 & 2) in Untreated Ehrlich Ascites Carcinoma Related to the Time of in vivo Pyknosis of the Tumour Cells. (Data from 15 ♂ & 15 ♀ Mice).

		Days before pyknosis*			Days after pyknosis*			
		9	6	3	3	6	9	12
Large injured tumour cells		Type 1			Type 2			
Mean %	♂	6.6	17.2	28.1	4.0	17.6	31.5	39.0
	♀	12.0	20.2	29.9	4.6	5.0	9.0	20.7
S.D.	♂	7.8	10.0	6.2	2.1	13.3	7.9	7.0
	♀	6.5	7.9	8.1	2.4	2.9	11.0	17.5
Number of biopsies	♂	8	13	15	13	9	6	2
	♀	3 §	5 §	9	15	14	12	8

* Maximum time possible. § Biopsy unsuccessful in one other mouse.

Table 1 shows the mean percentage of large damaged tumour cells, of both types, present in the biopsies preceding and following the occurrence of pyknosis. The standard deviation of these values and the number of biopsies studied is given. The results are shown graphically in Fig. 4.

In both sexes the number of injured tumour cells—type 1—rose to a peak in the biopsy preceding pyknosis and fell sharply when pyknosis had occurred. This drop in the number of type 1 cells is highly significant in both sexes ($0.001 > P$). After this time a further sex difference is apparent. The number of type 2 cells rose more quickly in the males than in the females; the differences in the two biopsies following the first showing pyknosis (6 and 9) being significant ($0.001 > P$).

DISCUSSION

The results of serial biopsy in the present experiment in which the number of injured tumour cells was seen to increase with time, and to reach a peak which was followed by pyknosis of the cells, are reminiscent of the findings on adding an excess of ascitic fluid to the tumour cells (*Hartveit* 1963a). In this previous experiment it was seen that when few large injured cells were present in vivo the addition of excess ascitic fluid increased their numbers and an even greater excess resulted in the pyknosis of the cells. The large injured cells produced were of the type seen in early biopsies in the present experiment—type 1 cells. The addition of even greater amounts of excess ascitic fluid did not give rise to type 2 cells as were seen in the later biopsies.

This indicates that although an increase in the amount of a cytotoxic factor may be responsible for the injury seen in type 1 cells it is unlikely to be the cause of the injury seen in the type 2 cells. This idea is under investigation and is supported by the morphology of the type 2 cells, in which the injury appears to effect both nucleus and cytoplasm simultaneously. In addition, as shown in Fig. 2b, the majority of the cells show minor changes of a similar nature, changes that are more like those seen on in vitro autolysis (*Hartveit* 1962b) than those due to immunological damage. It is suggested that these cell changes are probably due to anoxia, particularly as they were not seen until later biopsies when the amount of ascites was large and the host moribund.

A striking finding was that the biopsy with the greatest number of type 1 cells was invariably followed by a biopsy containing only a few injured cells, the rest being pyknotic. These pyknotic cells are undoubtedly viable. *Klein & Révész* (1953) found no difference in the amount of inoculum needed to produce an ascitic tumour from tumours between 3 and 16 days after transplantation. This is supported by the author's own experience that a 10 day tumour, in which the cells are usually pyknotic, is as reliable for transplantation as a 6 day tumour in which the cells are of normal appearance. In addition tumour cells

of similar pyknotic appearance produced by adding an excess of ascitic fluid to the tumour ascites, are viable (*Hartveit* 1963b).

It thus seems that after a critical percentage of injured tumour cells is present the remaining cells shrink and appear pyknotic. This critical percentage seems to be around 30 per cent, and to be the same in both sexes. The day this peak is reached varies greatly (presumably due to phenotypic and genotypic variations in the mice), and here there is a marked sex difference; the peak, and subsequent pyknosis, occurring before the 3rd. day in 6 of the females in contrast to the males (Fig. 2). This suggests that the immunological response occurs earlier in the females than in the males.

A further marked sex difference is that the number of large injured cells in the two biopsies following that showing pyknosis was lower in the females than in the males (Fig. 4). This is probably a reflection of the fact that pyknosis tended to occur earlier in the females and that the tumours were not so large by the time these biopsies were taken. Incidentally, this further supports the idea that the type 2 changes are the result of anoxia.

Unfortunately the cytological findings cannot be related to the survival time in this experiment as serial biopsy in itself may interfere with the survival time. Similarly the blood content of the tumour (*Hartveit* 1961b) cannot be measured reliably. However, the findings show that the greater immunity of female mice to the subcutaneous injection of Ehrlich's ascites carcinoma (*Hartveit* 1962a) is reflected in the morphology of the intraperitoneal tumour, in that the results of the immune response (*i.e.* increase in the number of injured cells and pyknosis) are seen earlier in female mice.

While the time of occurrence of the immune response is different in the two sexes the mechanism of the response itself appears to be the same; there are no significant differences in values obtained up to and including the first biopsy showing pyknosis, and it is probable that the time factor is also responsible for the differences after this time (vide supra).

SUMMARY

The morphological changes in Ehrlich ascites carcinoma cells were followed by serial biopsy. The number of large injured cells was found to increase with time until a critical percentage (30 per cent) was reached. Thereafter there were a few large injured tumour cells while the rest were pyknotic. Large injured tumour cells reappeared in later biopsies but were of different morphology from those preceding pyknosis. It seems likely that the injury in the latter cells is due to immunological damage while that in the former may well be the result of anoxia.

There was a marked sex difference in the mode of growth of the tumour in that pyknosis occurred earlier in the females. This supports

the author's previous findings (*Hartveit* 1962a and 1963a) that female mice have greater natural immunity to Ehrlich's ascites carcinoma than males.

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To be printed in
ACTA PATHOLOGICA ET MICROBIOLOGICA SCANDINAVICA

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THE SIGNIFICANCE OF THE PROTEIN CONTENT OF EHRLICH'S ASCITES CARCINOMA

By

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Received 14.iii.63

The possible presence of a cytotoxic factor in untreated Ehrlich ascites carcinoma has been demonstrated both in vivo and in vitro (*Hartveit* 1962 and 1963 a). It has been shown that swelling and loss of cytoplasm (similar to the cellular response to immunological damage in a low protein medium) is followed by pyknosis and clumping of the tumour cells. This pyknosis is not a more severe form of injury as the viability of the pyknotic cells is little reduced (*Hartveit* 1963 b). That is to say in early transplants there is evidence that the host defences are succeeding in injuring some of the tumour cells. After a certain time however, though there is still evidence that an immunological reaction is present—the cells are shrunken, clumped together and joined by intercellular bridges—there is also evidence that this reaction is abortive as the tumour continues to grow in spite of it (*Hartveit* 1963 b).

The question then arises as to why this immunological reaction is abortive. The reaction is a lytic one. Thus it is possible that lytic inhibitors may be involved. It is well known that the serum proteins may inhibit the lysis of erythrocytes in vitro, and probably also in vivo (*Ponder* 1948). On this basis it could be expected that an increase in the protein content of the medium surrounding the Ehrlich ascites carcinoma cells in vivo might inhibit lysis of these cells also. The finding that the number of injured tumour cells present increases with time in early transplants and is followed by a sudden change in the morphology of the tumour cells (which appear pyknotic) with no further evidence of lysis supports this idea, as the protein from the lytic cells could be expected to cause a rise in the protein concentration of the medium. If it is true that the protein in the medium inhibits the lytic reaction, the protein content should not rise once pyknosis has occurred.

The following experiments were designed to test this tentative hypothesis. In experiment I the appearance of Ehrlich ascites carcinoma cells from both early (prepyknotic) and late (pyknotic) transplants

was examined on phase contrast microscopy, both undiluted and in media of known protein concentration. As this experiment showed that tumour cells in a medium of low protein content could be recognized on phase contrast microscopy experiment II was set up to apply this finding to the tumour as it is in vivo. Experiment III was designed to see if the protein content of the medium rose after the tumour cells had become pyknotic.

MATERIAL AND METHODS

The mice used and the Ehrlich ascites carcinoma were similar to those used in previous experiments (*Hartveit* 1961), tumour from the 135th and 141st transplant generations being used.

TABLE 1

Ehrlich's Ascites Carcinoma—the Types of Tumour Cells in the different Transplants.

Type of transplant	Prepyknotic	Early pyknotic	Pyknotic
Types of tumour cells	Normal and Type 1*	Pyknotic and Type 1*	Pyknotic and Type 2*

* See text.

It has been shown previously (*Hartveit* 1963 c) that the type of tumour cell present in Ehrlich's ascites carcinoma varies with the time after transplantation. Thus it is possible to classify the transplants into three types as is shown in Table 1. Firstly there are the transplants in which pyknosis has not yet occurred; these prepyknotic transplants contain normal tumour cells and large tumour cells showing injury of an immunological type (type 1 cells). Secondly there are the early pyknotic transplants which contain pyknotic tumour cells and a few type 1 cells. Then, thirdly, there are the pyknotic transplants which contain pyknotic tumour cells in the early stages. When the host is moribund these transplants also contain autolytic (type 2) cells. (See Figs. 1 and 2 in *Hartveit* 1963 c).

On examining preparations of Ehrlich's ascites carcinoma cells under phase contrast microscopy it must be remembered that the nucleus of the cell is large and that the cytoplasm is scanty. The light halo may also be disturbing—especially on photographs. The comparison to be made in the following experiments is between the narrow rim of cytoplasm and the medium in which the cells are suspended. This is illustrated diagrammatically in Fig. 1.

Experiment I.

Five prepyknotic and 5 pyknotic tumours were obtained by collecting the tumour ascites from male and female mice that had each received an intraperitoneal injection of 0.1 ml of Ehrlich's ascites carcinoma (tumour cell count 1,180,000/mm^3, tumour blood content—1 per cent), three or twelve days previously.

Wet preparations of the undiluted tumour were examined at once by phase contrast microscopy. Then one part of the tumour ascites was diluted with 10 parts of a solution of 1 g per cent bovine albumin in phosphate buffered physiological saline (a low protein medium). A further one part of the tumour ascites was diluted with 10 parts of a similar solution containing 2.4 g per cent bovine albumin (a high protein medium). Wet preparations of the cells in these media were examined at once by phase contrast microscopy.

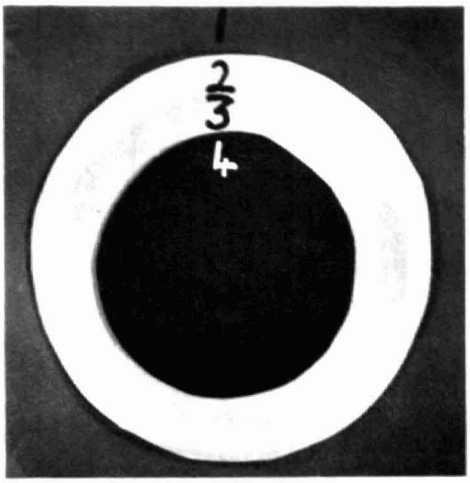


Fig. 1.
Diagrammatic representation of an Ehrlich ascites carcinoma cell on phase contrast microscopy.
1 – medium. 2 – light halo. 3 – cytoplasm. 4 – nucleus.

Experiment II.

One male mouse that had been given 0.1 ml of Ehrlich's ascites carcinoma 10 days before provided the tumour for the two experimental groups (A and B) each of which consisted of 5 male and 5 female mice. Each of these was given 0.1 ml of the tumour ascites intraperitoneally (tumour cell count 1,290,000/mm^3, tumour blood content—2 per cent). The mice in group A were killed two days after transplantation and those in group B eight days after. In all cases the tumour ascites was then removed—films made and stained, as described previously (*Hartveit* 1962), and wet preparations examined at once by phase contrast microscopy.

Experiment III.

One male mouse that had been given 0.1 ml of Ehrlich's ascites carcinoma intraperitoneally 10 days before provided the tumour for the two experimental groups (C and D). Group C consisted of 10 male and 10 female mice, group D of 15 male and 15 female mice. Each of these was given 0.1 ml of the tumour ascites intraperitoneally (tumour cell count 1,290,000/mm^3, tumour blood content—a trace). The survivors in group C were all killed on the 8th day following transplantation—those in group D on the 12th. When the mice had been killed the tumour ascites was removed. Films were made from this and stained as in the previous experiment. The tumour ascites was then centrifuged and the protein (plus nucleic acid) content of the cell-free ascitic fluid estimated spectrophotometrically, using a Unicam spectrophotometer and an extinction of 280 μm.

RESULTS

Table 2 gives a summary of the results.

Experiment I.

The findings are further illustrated in Figs. 2 and 3. The cytoplasm of the tumour cells was dark in the undiluted prepyknotic tumours and in the same cells suspended in a medium of known low protein content.

TABLE 2

Summary of Results.

The Appearance of Ehrlich's Ascites Carcinoma Cells on Phase Contrast Microscopy in Media of different Protein Content. Low Protein Content—LPC, High Protein Content—HPC.

Experiment	Type of tumour	Medium	Cytoplasm	Type of tumour cell	Note
I	Prepyknotic	LPC	Dark	Normal + type 1	See Fig. 2 a.
		HPC	Bright	Pyknotic	See Fig. 2 b.
		Undiluted tumour	Dark	Normal + type 1	
	Pyknotic	LPC	Bright	Pyknotic	See Fig. 3 a.
		HPC	Bright	Pyknotic	See Fig. 3 b.
		Undiluted tumour	Bright	Pyknotic	
II	Prepyknotic	Undiluted tumour	Dark	Normal + type 1	
	Early pyk.	Undiluted tumour	Dark & bright	Pyknotic + type 1	
	Pyknotic	Undiluted tumour	Bright	Pyknotic	

Note: No sex differences were observed.

In all other cases it was bright. Type 1 cells were only present when it was dark.

Experiment II.

Stained films. Table 3 shows that the type of tumour varied in the two groups. At two days most of the tumours were prepyknotic while at 8 days nine out of ten of the tumours had reached the pyknotic stage. The sex differences in the findings are not statistically significant, but the differences between the groups are ($0.01 > P > 0.001$). These results are in keeping with the results of serial biopsy reported earlier (*Hartveit* 1963 c).

TABLE 3

The Type of Tumour Present in Mice with Intraperitoneal Ehrlich's Ascites Carcinoma Related to Time after Transplantation. (Leishman's Stain.)

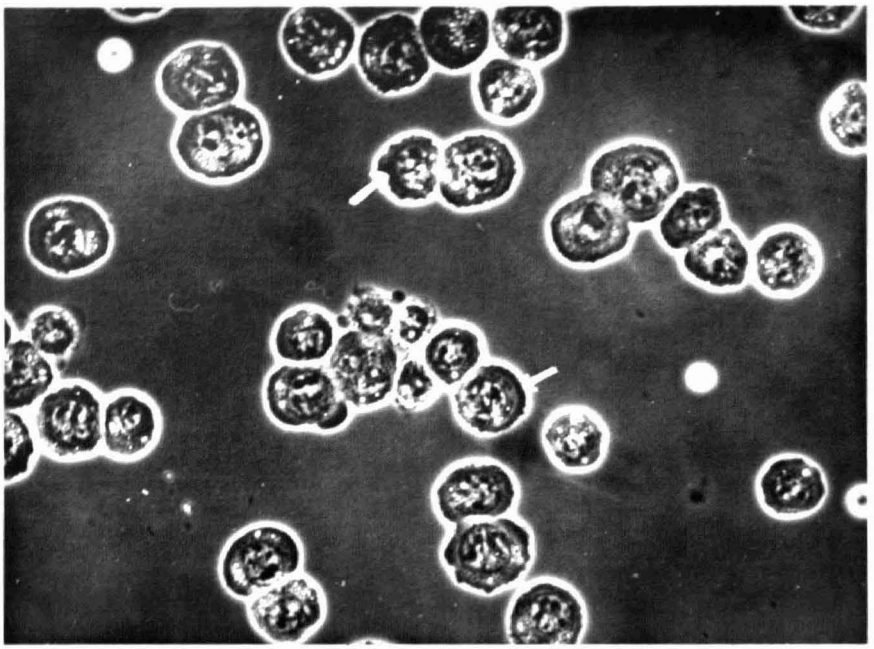
Group	Days after transplant.	Type of tumour		
		Prepyknotic	Early pyk.	Pyknotic
A	2	4♂ + 4♀	1♂	1♀
B	8	0	1♀	5♂ + 4♀

Phase contrast microscopy. When these results were compared with those from the stained films it was found that in the cases where the tumours were at the prepyknotic stage (Fig. 4 a) the cytoplasm of the cells was darker than the medium, in the early pyknotic stage the cytoplasm was dark in some and light in other cells, while in the pyknotic tumours (Fig. 4 b) the cytoplasm was brighter than the medium in all cases (Table 2). Once again type 1 cells were only present when the cytoplasm was dark. Thus in the light of the findings in experiment I the medium surrounding the tumour cells in prepyknotic transplants is probably of low protein content. This is supported by *Ledoux & Revell*'s (1955) findings with the Landschütz tumour in which the protein content of the ascitic fluid was lowest in early transplants.

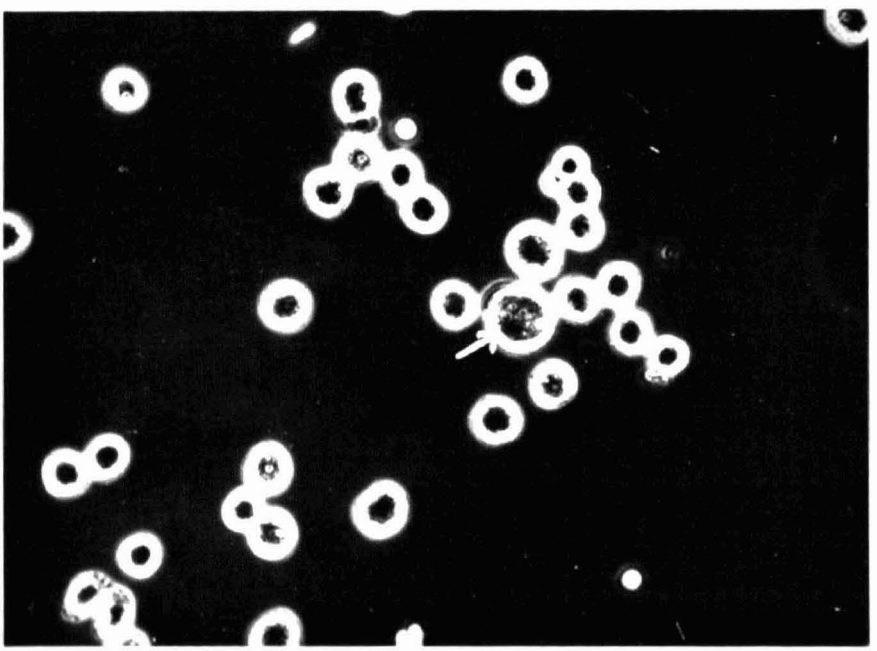
Experiment III.

At eight days there were 8 male and 10 female survivors in group C, and at twelve days 11 male and 8 female survivors in group D. In group C the tumour cells had reached the pyknotic stage in all but three of the males. As the experiment was designed to find the protein content of the pyknotic tumours these three animals were excluded from further investigations. All the tumours in group D were pyknotic, a few autolytic cells were present in some cases.

Table 4 shows the mean protein content (g per cent) of these pyknotic tumours, with its standard deviation (S.D.). The differences between the groups and between the sexes are not statistically significant.



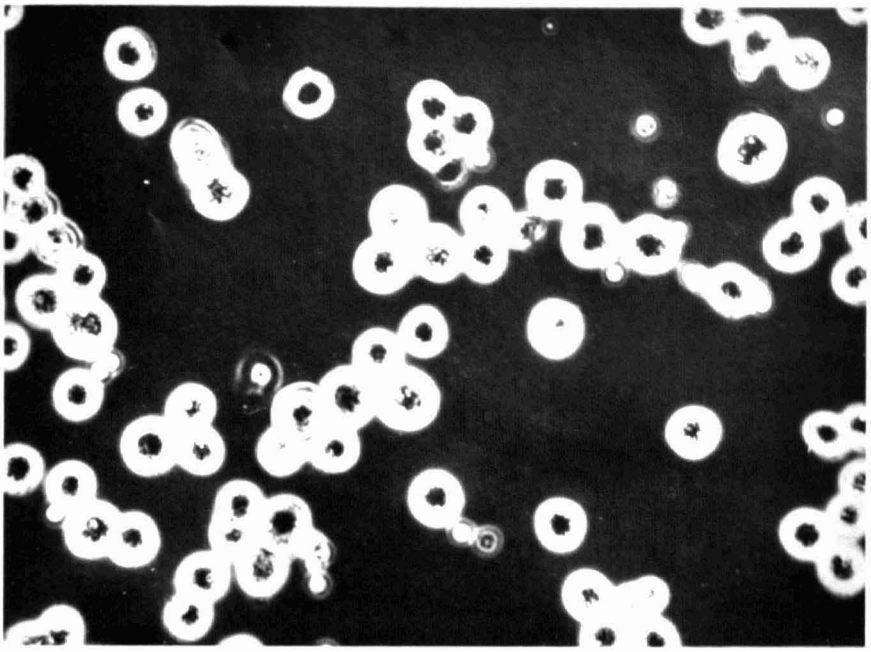
a. Cells suspended in low protein medium. Note darkness of cytoplasm (→).



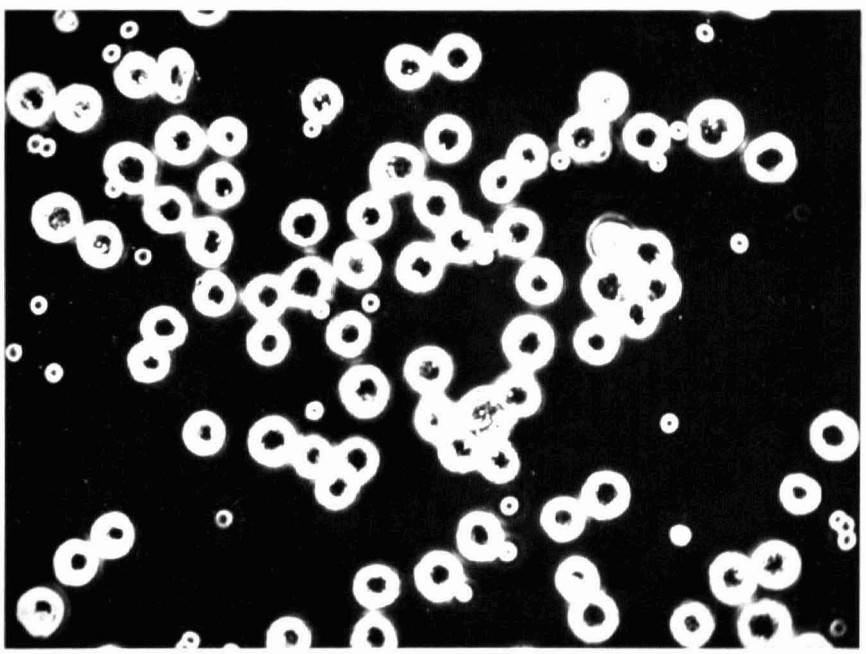
b. Cells suspended in high protein medium. Note brightness of cytoplasm (→).

Fig. 2.

Ehrlich ascites carcinoma cells from a *prepyknotic* transplant suspended in media of different protein concentration. (Phase contrast microscopy × 400).



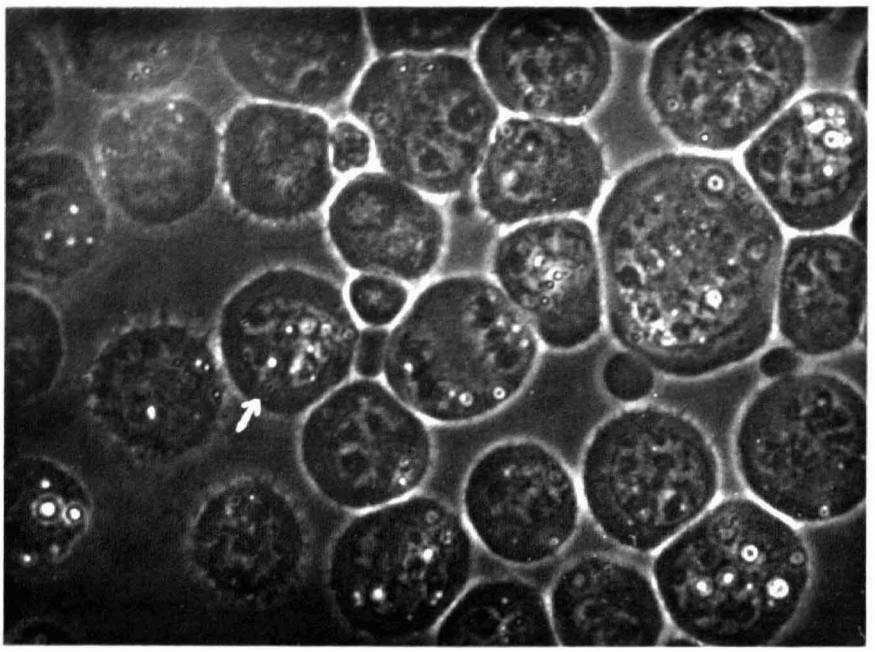
a. Cells suspended in low protein medium. Note brightness of cytoplasm.



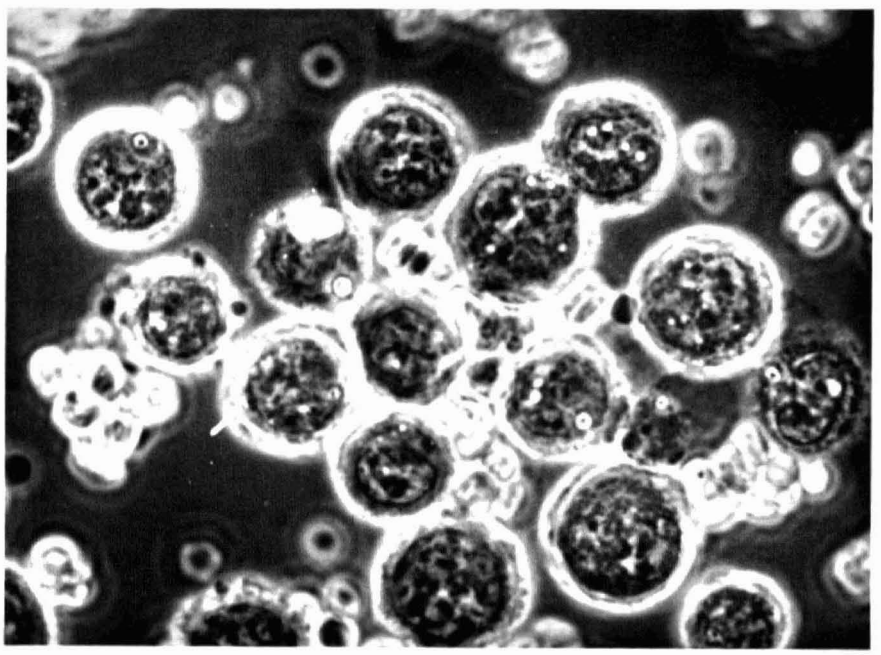
b. Cells suspended in high protein medium. Note brightness of cytoplasm.

Fig. 3

Ehrlich ascites carcinoma cells from a *pyknotic* transplant suspended in media of different protein concentration. (Phase contrast microscopy × 400).



a. A prepyknotic transplant. Note darkness of cytoplasm (→), and particularly of cytoplasmic blebs.



b. A pyknotic transplant. Note brightness of cytoplasm (→).

Fig. 4.

The appearance of Ehrlich ascites carcinoma cells on phase contrast microscopy. (Undiluted specimen, × 1100).

These results show that the protein content of the medium in these pyknotic tumours is high and that after the tumour has reached the pyknotic stage the protein content of the medium does not increase. These findings are in keeping with those of *Kun, Talalay & Williams-Ashman* (1951) from pooled tumour ascites between 7 and 12 days after transplantation. *Burgess & Sylvén* (1962) give slightly higher values, 3–4 g per cent, but these were for a distinct subline of the tumour.

TABLE 4

The Mean Protein Content ($\bar{x}$), plus S.D., of the Medium Surrounding Pyknotic Ehrlich Ascites Carcinoma Cells in vivo at 8 and 12 Days after Transplantation.

Group	Days after transplant.	Series	No. of mice	$\bar{x}$ (g%)	S. D. $\bar{x}$
C	8	♂	5	2.76	0.65
		♀	10	2.57	0.89
D	12	♂	11	2.55	0.28
		♀	8	2.57	0.31

DISCUSSION

Experiment I of the present paper shows that it is possible, using phase contrast microscopy, to say whether an Ehrlich ascites carcinoma cell from a prepyknotic transplant is in a medium of high or low protein content, as defined above. The cytoplasm of the cells from these transplants appeared dark in media of low protein content and bright in media of high protein content. On the other hand, the cytoplasm of cells from pyknotic transplants appeared bright regardless of the protein content of the medium. Thus while darkness of the cytoplasm can be taken as evidence that these cells are in a medium of low protein content, the converse—that cells in which the cytoplasm is bright are in a high protein medium—is not necessarily true.

Experiment II makes use of these findings to confirm that the medium in prepyknotic transplants is of low protein content, as the cytoplasm was dark in all cases. The volume of these early transplants is also low which makes direct estimation of the protein content difficult if pooled samples are to be avoided. In addition other methods of estimating the protein content of the medium involve centrifugation to remove the tumour cells. This process breaks up the type 1 cells present and renders subsequent estimations of the protein content of the medium meaningless. (This objection to centrifugation of the samples does not hold in experiment III as type 1 cells were not present and pyknotic cells do not show morphological damage on centrifugation). The cytoplasm of the pyknotic cells was bright in all cases, but no conclusions as to the protein content of the medium can be drawn from this finding. However, experiment III showed that the protein content

of the medium in pyknotic transplants is high and, in addition, that it does not increase with time.

It is suggested that the increase in the protein content of the medium surrounding the cells that must take place if, as has been shown previously (*Hartveit* 1963 c), a prepyknotic tumour proceeds to become a pyknotic one, is the result of loss of protein from the type 1, the immunologically injured, cells. It appears from previous results (*Hartveit* 1963 c) that when about 30 per cent of the tumour cells have lost their cytoplasmic protein to the medium the remaining tumour cells become pyknotic. From that time on no further loss of protein to the medium takes place, as is shown in experiment III. While only 30 per cent of the tumour cells show signs of gross injury all the remaining cells shrink, show clumping and intercellular bridge formation (*Hartveit* 1963 c). Thus all the cells show evidence of response to an immunological reaction although lysis does not result in all cases. This is of note in view of *Flax's* (1956) and *Lindner's* (1960) reports that about 70 per cent of the tumour cells are insensitive to the effects of tumour specific antiserum.

The above findings suggest that the high protein content of the medium surrounding the pyknotic tumour cells protects them from damage of an immunological type. Green, Barrow & Goldberg (1959) have previously shown that such damage takes place in media of low protein content (about 0.5 g per cent), and this is in keeping with the findings in the present experiment. But their findings in media of a "high" protein content, which have been confirmed by *Agol* (1961), cannot be compared with the findings in the present experiment as the "high" protein content is different. In their work the high protein content was between 15 and 20 g per cent, a very unlikely situation physiologically. They argue that swelling of the antibody treated tumour cells, in the presence of complement, was prevented osmotically in their high protein medium. In the present experiments osmotic prevention of swelling cannot explain the results. It seems more likely that the lytic reaction is inhibited by the proteins that are present in physiological amounts. Here *Amos's* (1961) findings are of note, as he has shown that in some homografts (L 1210 in C3H mice), the tumour population becomes sensitized during the time of rejection and that sensitized cells tend to accumulate faster than they can be eliminated by phagocytosis or by lysis. He goes on to state that "many of the cells are stained immediately by Trypan blue," (i.e. are of doubtful viability), "others after incubation in Ringer's solution". In other words more cell damage became evident when the protein content of the medium was reduced.

It may be objected that the use of bovine albumin in the protein media used in the tests is artificial and that mouse protein might not act in the same way. However, further control experiments have shown that the cytoplasm of Ehrlich's ascites carcinoma cells from both pre-

pyknotic and pyknotic transplants appears bright in undiluted serum from normal mice, while if the serum is diluted 1:10 with physiological saline the prepyknotic and pyknotic cells both show dark cytoplasm. The finding that the pyknotic cells also develop dark cytoplasm is of note, as this change is similar to that which occurs in vivo on transplantation. As was shown in experiment I lowering of the protein content of the medium alone is not enough to bring about this change. This suggests that mouse serum may contain a further factor that is needed to convert a pyknotic cell to a prepyknotic one. In view of common experience with tumour specific antiserum in vitro it may be one of the components of complement that is needed. If so this factor must be lacking in late transplants or the cytoplasm of the pyknotic cells would have become dark on reduction of the protein content of the medium. Conversely, enough of this factor must be present in prepyknotic transplants to allow the immunological reaction to take place. It is unlikely that lack of complement limits the reaction in vivo as the cytoplasm of the prepyknotic tumour cells diluted in a high protein medium appeared bright, while those diluted in a low protein medium appeared dark. As the amount of complement would have been the same in both cases it cannot be this that determines the appearance of the cytoplasm, or the type of tumour cell present. Thus the survival of the Ehrlich ascites carcinoma cells in the presence of the cytotoxic factor may well be due to the protective effect of the protein content of the ascitic fluid.

SUMMARY

On the basis of the findings on phase contrast microscopy it was shown that the protein content of the ascitic fluid surrounding Ehrlich ascites carcinoma cells in vivo is low in prepyknotic transplants. The protein content of the fluid in pyknotic transplants is high (about 2.5 g per cent). As cell damage of an immunological type is only seen in prepyknotic transplants the results of these experiments support the hypothesis that the proteins in the ascitic fluid may, by acting as a lytic inhibitor, protect the tumour cells from the action of the cytotoxic factor produced by the host in response to the homotransplanted tumour.

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SUMMARY AND GENERAL DISCUSSION

As mentioned in the introduction the present work started as an attempt to induce immunity to the Ehrlich ascites carcinoma. This tumour was thought to grow progressively in all mice due to its lack of antigenicity — being an exception to the general rule that a homograft will be rejected by the host. At first sight it seemed that these attempts to induce immunity failed. But in the course of these preliminary experiments it became clear that the blood content of the tumour ascites — a factor that had hitherto been dismissed as a contaminant — might be of significance.

This blood could only have come from the host. Therefore it represents, either directly or indirectly, a host response to the tumour. The finding that the blood content of the tumour ascites differed from mouse to mouse prompted the experiment reported in the first paper in this series. This was an attempt to find the conditions that normally obtained when Ehrlich's ascites carcinoma was transplanted intraperitoneally in the mice used at this Institute. It was found that the blood content of the tumour was higher in mice that died soon after transplantation than in the long survivors. It also became clear that the mice used were of two main types — those that died soon after transplantation and those that survived a considerably longer time. As it was felt that the only variable factor in this experiment could be the genetic constitution of the mice, it seemed possible that some of the mice might, contrary to expectation, be reacting against the tumour transplant. The results of the final experiments designed to test this hypothesis are reported in paper II. On using means aimed at reducing any immune response that might be present it was found that the blood content of the tumour was reduced. This was interpreted as an indication that the blood content in itself might be a measure of such a response to this tumour. Consequently the mice that died soon after the injection were considered to be dying of the effects of their reaction against the tumour.

The original experiments in which living tumour was combined with Freund's adjuvant and given subcutaneously to mice with intraperitoneal tumour were then repeated as the blood content of the intraperitoneal tumour could now be used to measure the host response (papers III and IV). Control groups without intraperitoneal tumour, with subcutaneous adjuvant alone and with no subcutaneous treatment were also studied. It was found that the natural immunity of the host to the tumour was increased in the experimental group and, in addition, in some cases immunity appeared to have been acquired. The findings also indicated that the mechanism of the immune reaction was probably the same in natural immunity and in acquired immunity produced in this way. The mice given subcutaneous adjuvant alone and intraperitoneal tumour showed immunity of a non-specific type the mechanism of which seemed to differ from that of natural and acquired immunity.

It was further shown that female mice have greater natural immunity to this tumour than males, and that immunity is easier to induce in females. Haemolysis of the blood in the tumour occurred in the mice given tumour plus adjuvant. This may have been a response to the erythrocytes in the subcutaneous tumour adjuvant mixture.

It was now clear that the original assumption that the mice do not react against the progressively growing tumour was no longer tenable and that the transplant was in fact recognized as a homograft, at least by some of the mice. This being so it would be reasonable to expect some kind of cellular injury in the haemorrhagic tumour transplants. The investigations in paper V were designed with this in mind and it was found that cellular injury was present at six days after transplantation in *all* transplants. The number of injured cells was consistently greater in the haemorrhagic than in the non-haemorrhagic tumours, but were present in both. Thus it was concluded that not only the animals with haemorrhagic but also those with non-haemorrhagic tumours were reacting against the transplanted tissue.

These findings, and the observations that cell injury started on the cell surface and that the cytoplasm was injured before the nucleus showed signs of damage, suggested that the injurious factor might be present in the ascitic fluid surrounding the tumour cells. It was found, as reported in paper VI, that such was probably the case, as the addition of an excess of ascitic fluid to the tumour cells increased the number of injured cells present, while further excess resulted in shrinkage of the cells with condensation of the cytoplasmic and nuclear proteins — i.e. pyknosis. (These cells are termed "pyknotic" in the present work, and may be similar to those described by Amos (2) that "became smaller and stained more intensely with less definition of nuclear chromatin" in the course of a homograft reaction.) The ascitic fluid from haemorrhagic tumours was found to be richer in this injurious factor than that from non-haemorrhagic tumours — i.e. less fluid from the former was needed to produce cell damage.

It was first thought that the pyknosis referred to above was similar to that seen on autolysis and also described by King et al. on irradiation and on treatment with a mercurial poison. The experiment described in paper VII was set up to see if this was the case. The result was the opposite of that expected — the so-called pyknotic tumour cells were viable. Thus they cannot be cells that have burst in response to injury — but must be cells that have shrunk without first bursting. In the light of the above findings it was next decided to investigate the tumour cells by serial biopsy (paper VIII). This disclosed that the type of cell damage described previously at six days increased to a peak when about 30% of the tumour cells showed these changes. This peak was followed by an abrupt change in the morphology of the tumour cells — the next biopsy showing predominantly cells of the type seen after the addition of large amounts of excess ascitic fluid to the tumour cells in vitro — i.e. pyknotic cells. Thus it became evident that the cycle of events seen in vitro on adding excess ascitic fluid to the tumour cells parallelled the natural occurrences in the life history of the tumour in vivo.

At this stage in the experiments it was clear that, although an immunological type reaction against the tumour cells was present, the tumour grew in spite of it. It seemed possible that the pyknotic cells in the late transplants might be antibody coated cells, but that cytolysis was being prevented in some way.

On the basis of analogy to the inhibitory effect of serum proteins on lytic reactions (48) the protein content of the ascitic fluid round the tumour cells was studied. These experiments are reported in paper IX; the conclusion reached being that the suspicion was justified and that, although complement may also be lacking in some cases, it is probably the protein content of the ascitic fluid that protects the tumour cells from the destruction that should be their lot on homotransplantation.

This survey of the present work has been included to explain the way in which these experiments came to be done — how one aspect of the question was often followed to the exclusion of another — in other words to show the train of thought that lies behind the work. As will have been seen this train of thought was originally based on conventional ideas in the field of tumour immunity. One of these ideas — the concept that a tumour such as the Ehrlich ascites carcinoma is transplantable from mouse to mouse irrespective of breed because it lacks antigens the mice can recognize as foreign — has been shown to be incorrect in this case. The present work also shows that the Ehrlich ascites carcinoma and the host reaction to it follow the basic rules of transplantation immunity. The tumour is recognized by the host which then mobilizes its defences with the production of an immune reaction. However, the clinical result — rejection of the transplant — does not take place as the protein in the medium in which the cells are suspended in vivo protects the potentially damaged cells from the lytic action of the cytotoxic factor plus complement. As swelling with consequent disorganization of cell structure and cell function is prevented the antibody coated cells continue to live unhampered by their hostile environment. When the above findings are borne in mind it is not surprising that antibodies are not usually found in the serum of mice with Ehrlich's ascites carcinoma, as it may well be that all the antibody is adsorbed to the tumour cells as it is produced.

While it must be remembered that a spontaneous tumour and a transplantable tumour such as the Ehrlich ascites carcinoma are basically different, as the latter is a homograft, it must not be forgotten that the Ehrlich ascites carcinoma was originally a spontaneous tumour and that the clinical course in the animal bearing the tumour is similar in both cases. In both cases the tumour grows progressively and may metastasize. In both cases it will eventually kill the host. In neither case can the animal bearing a progressively growing tumour be shown to have immunity to a further transplant of the same tumour, and reports of serum antibody or delayed sensitivity to such tumours are exceptional.

As stressed previously lack of antigenicity was thought to explain the progressive growth of Ehrlich's ascites carcinoma — but this has been shown to be incorrect. The lack of response to spontaneous tumours has also been put down to lack of antigenicity — but now tumour specific antigens have been demonstrated in some tumours and recent advances in the technique of gel diffusion and the discovery of immunological tolerance make it likely that more will soon be uncovered.

It is tentatively suggested that the similarities between the Ehrlich ascites carcinoma and spontaneous tumours should be considered in assessing the position of the latter in the field of tumour immunity. Both were thought to be exceptions to the general rules. Ehrlich's ascites carcinoma was considered to be a homograft that, against the rules, does not regress. A spontaneous tumour is thought to carry a mutant, or changed, gene, that in theory should turn it

into a "homograft" — i.e. a tissue of the same species that differs genetically from the host — but, once again against the rules, such a tumour does not regress. In the introduction to the present work it was suggested that the lack of clinical and serological evidence of immune response to spontaneous tumours might not be due to failure of the tumour to elicit the response but to failure of the reaction to go to completion — i.e. to result in tumour cell damage.

This hypothesis is strengthened by the author's findings concerning the Ehrlich ascites carcinoma in the present work. If the response to such a tumour can be abortive it is possible, on the basis of the similarities outlined above, that such an explanation might also hold for spontaneous tumours; these may also grow in spite of the host response and not due to lack of host response. If it is the protein in the medium surrounding the tumour cells that protects them from the results of immunological damage it is even more likely that this protective mechanism would work with solid tumours as it has been shown that the interstitial fluid from solid tumours is of higher protein content than the fluid from some ascitic tumours (6), in particular the Ehrlich ascites carcinoma, while that of blood plasma is higher still.

Lack of complement could also explain the failure of the lytic reaction. But if this is the case it must be postulated that complement is lacking from the start of tumour growth. While the host might well become depleted of complement in time, as antibody was adsorbed to the tumour cells, there is no reason to believe that complement is not present originally. If complement is present at the start of tumour growth it is unlikely to be the limiting factor in the immunological reaction against the tumour as the tumour cells would, theoretically, not survive in its presence. Thus, although complement may well be lacking in advanced tumour growth, it seems more reasonable to attribute the initial failure of the reaction to the inhibitory action of the extracellular proteins.

It is suggested that it may be a balance between the amount of tumour present, the amount of specific antibody, the protein content of the medium and the availability of complement that determines the fate of tumour cells. These ideas are necessarily fragmentary and of a highly tentative and speculative nature. The ramifications of such a hypothesis are extensive and much work will be needed before its usefulness can be evaluated, particularly in therapeutics, but it offers the possibility of widening our views on tumour immunity and maybe, ultimately, understanding it better.

GENERAL CONCLUSIONS

1. The blood content of the Ehrlich ascites carcinoma varies with the survival time of the mice which shows a bimodal distribution. Animals with a high tumour blood content die earlier than those in which the tumour blood content is low. Within the limits of the experiment the sex and weight of the mice do not effect their survival time.
2. As the blood content of the tumour can be reduced markedly by methods known to reduce the immunological response, the blood content is probably a reflection of the host response to the tumour homograft.
3. The subcutaneous injection of Freund's adjuvant combined with living tumour both increases the natural resistance of the mice to the tumour and may also induce immunity to it. Adjuvant alone results in non-specific immunity which appears to differ in its mode of production from natural and from acquired immunity.
4. Female mice appear to have greater natural resistance to the tumour than males. The non-specific immunity resulting from the injection of adjuvant alone becomes evident earlier in the females. Haemolysis seen in the tumour following the injection of adjuvant and whole tumour ascites may be a result of a reaction against the erythrocytes in the whole tumour.
5. Tumour cell injury — of an "immunological" type — is seen in early transplants of this tumour and is directly related to the tumour blood content.
6. The ascitic fluid surrounding the tumour cells, if added to the whole tumour in slight excess, results in tumour cell lysis. The addition of still further excess leads to pyknosis of the cells.
7. These pyknotic tumour cells — though they show evidence of an immunological reaction — are viable.
8. Changes similar to those seen in vitro on the addition of ascitic fluid to the whole tumour occur in vivo; lytic cells being present in early, and pyknotic cells in late transplants. Females show evidence of immune response earlier than males.
9. The protein content of the ascitic fluid surrounding the tumour cells in vivo is low (about $1g^0/_0$) in early transplants and high (about $2.5g^0/_0$) in late transplants.
10. The Ehrlich ascites carcinoma can no longer be considered non-antigenic in mice as host response is not lacking. This response is at first successful in the rejection of the tumour cells — but before all the tumour cells have been destroyed the protein content of the ascitic fluid rises and inhibits the final stage of the immunological reaction — cell lysis.
11. Thus the tumour is able to grow in spite of the host response. This mechanism of protection may be of wider application. An autogenous tumour, if it possesses antigens not present in the host, could be likened to a homograft. In that case lack of rejection of tumour growth may not be an indication of lack of recognition, but an expression of immunological tolerance of the type described in the present work.

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* (Note: This is not intended as a review of the literature as such would be superfluous at present, but as an indication of where further references are to be found.)

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