phosphatase staining granules. One group consisted of large cells containing granules which stained after only 5-min incubation with the substrate mixture. These would appear to be cells of the macrophage series and were similar to cells in the peritoneal exudate which had ingested oil; the cytoplasm of these cells was not basophilic. A second group of large cells which had an intensely basophilic staining cytoplasm and corresponded to the large pyroninophilic cells in previous studies[3,4] had a much smaller number of granules which only began to stain for acid phosphatase after 20 min incubation with the substrate mixture. Finally, there were small lymphocytes which contained granules which stained optimally for acid phosphatase following 2-h incubation. As can be seen in Table 1, there was a marked increase in the proportion of small lymphocytes containing lysosomes in the lymph nodes draining an area of application of a chemical sensitizing agent. The average number of lysosomes per cell also increased, reaching a peak 4 days after sensitization, falling off on the sixth day after sensitization.

Table 1. Effect of Sensitization to Oxazolone on the Activity of Lysosomes in Lymphocytes within the Draining Lymph Node

Auricular lymph node	Small lymphocytes: Maximum percentage of cells containing lysosomes	Small lymphocytes: Mean number of lysosomes per cell	Large lymphoid cells: Per cent cells containing lysosomes
Normal	10	3	19·5
2 days after sensitization	78	6·3	77
4 days after sensitization	89	8·1	93·5
6 days after sensitization	85	5·1	77·5

All figures are the mean of those found on examination of the material from six different animals. The 'large lymphoid cells' were found to have a strongly basophilic cytoplasm. Highly active macrophages which were not basophilic were not included in this series.

About 35 per cent of small lymphocytes in the peripheral blood or an oil-induced peritoneal exudate from normal guinea-pigs were found to contain lysosomes (Table 2). In guinea-pigs which had been sensitized with dead tubercle bacilli in water-in-oil emulsion 3–4 months previously, the number of small lymphocytes containing lysosomes had increased to between 80 and 90 per cent. Cells from sensitized animals contained about 10–11 lysosomes per cell as compared with 3·5–5 lysosomes per cell in normal animals. Lysosomes in small lymphocytes from sensitized animals were seen to be markedly swollen when compared with those from normal animals. Incubation of cells from peritoneal exudates with the substrate mixture for only 5 min showed that macrophages from tuberculin-sensitive animals had more active lysosomes than macrophages from normal animals.

Table 2. Effect of Sensitization with Tubercle Bacilli on the Activity of Lysosomes within Small Lymphocytes in the Peripheral Blood and in a Peritoneal Exudate

Source	Normal guinea-pigs: Per cent active cells	Normal guinea-pigs: Mean No. of lysosomes per cell	Tuberculin sensitized guinea-pigs: Per cent active cells	Tuberculin sensitized guinea-pigs: Mean No. of lysosomes per cell
Peripheral blood	36 (3)	5·1	80·6 (3)	11·5
Peritoneal exudate	35·2 (5)	3·5	92·2 (5)	9·8

Figures in parentheses indicate the number of animals examined.

In an investigation of this type it is important to differentiate cells of the lymphocyte series from macrophages. Macrophages can be distinguished easily because their lysosomes appeared to have a more fragile lysosomal membrane and consequently the lysosomes stain for acid phosphatase after a shorter incubation time than cells of the lymphocyte series. Lymphocyte lysosomes would appear to be more stable than those of macrophages. Large cells of the lymphocytes series with a basophilic cytoplasm (immunoblasts) were seen in lymph nodes draining the area of application of a chemical-sensitizing agent, reaching a peak in concentration on the fourth day after sensitization. A high proportion of these cells contained lysosomes. However, these lysosomes were present in smaller numbers and were far less fragile than those seen in macrophages. These cells would appear to be similar to those described by previous authors[1,2] in studies on the lysosome content of lymphoid cells in tissue cultures containing phytohaemagglutinin.

On the days immediately following the application of a chemical sensitizing agent to the skin, there was a marked increase in the number of small lymphocytes containing lysosomes in the draining lymph node. The number of active lysosomes in an active cell also increased, reaching a peak on the fourth day after sensitization. It is not known whether these cells were precursors of the immunoblasts, or were derived from them by cell division. However, they were still present in large numbers on the sixth day after sensitization, at a time when it was known that the number of immunoblasts had fallen considerably[3,4]. It would therefore be unlikely that these cells were about to differentiate into immunoblasts.

It may also be that small lymphocytes containing lysosomes are immunologically competent cells. It is therefore interesting that there is an increased number of small lymphocytes with lysosomes in animals injected with tubercle bacilli in water-in-oil emulsion and that these are also much larger in size and increased in number. This may also have some bearing on the increased immunological responsiveness of animals injected with Freund's adjuvant. One might also speculate on whether the small lymphocytes which contain lysosomes are the effector cells in delayed-type hypersensitivity. However, in preliminary experiments in which tuberculin has been injected intraperitoneally in a dose sufficient to cause aggregation of macrophages (10 μg PPD)[9] no significant change was found in the lysosomes of either small lymphocytes or macrophages.

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Precocious Anti-diphtheria Response induced by RNA Immuno-carrier

In previous papers an increase of the RNA content in the serum of rabbits immunized with horse serum albumin and rat or guinea-pig red blood cells was observed; the greatest increase was in the gamma-globulin fraction. The RNA extracted from immune rabbit sera (RNA Immuno-Carrier or RNA-I-C) was able to induce in normal rabbits a precocious antibody production against the same antigens used for immunizing the animals' source of the RNA[1,2].

It was then found that RNA-I-C from the serum of animals immunized with heterologous RBC was able to induce rapid antibody production in normal animals of different species[3]. The possibility was also demonstrated of obtaining a precocious auto-haemantibody response in normal rats treated with a suitable amount of RNA-I-C from serum of rabbits immunized with rat RBC[4].

The following investigations were undertaken to ascertain whether it is possible to induce a precocious antibody response in normal animals with RNA from serum of animals immunized against diphtheria toxin. Further-

more, because in preliminary experiments an attempt to obtain an effective RNA-I-C from 'stored' antitoxic horse serum was unsuccessful, we tested whether the RNA content of 'stored' immune sera was changed, and whether the capacity of inducing immunity in normal animals was modified in RNA extracted from 'stored' immune sera.

Female rabbits weighing 1·8–2·0 kg, fed on a standard diet, were immunized with 200 Lf of diphtheria toxoid ('Anadifterall' Sclavo) given by four intravenous injections at four-day intervals; seven days after, a fifth injection of 50 Lf was made and, nine days afterwards, the blood of the animals was collected. One part of the serum was 'stored' at +2° C for 40 days, the remainder was used at once.

The RNA was estimated and extracted from anti-diphtheria rabbit sera 'fresh' or 'stored', according to the technique described in a previous paper[2]; as a control, anti-diphtheria horse serum (from Sclavo, Siena, Italy) 'stored' for 40 days was used as a source of RNA.

The RNA so obtained was administered by a single intravenous injection to normal rabbits, 1·5 mg/kg body-weight; the animals were bled 48 h after the RNA injection and the sera were used in the diphtheria toxin neutralizing tests.

Four groups of male guinea-pigs weighing about 250 g (with the abdominal skin previously shaved) were used: to the first group was given subcutaneously, in a final volume of 4 ml., a mixture of various amounts of diphtheria toxin (1 ml. = 1,000 MLD; from Sclavo, Siena, Italy) corresponding to 1; 2·5; 5; 7·5; 10; 15; 20 MLD, pre-incubated for 1 h at 37° C with 0·05 ml. of serum from rabbits treated with RNA-I-C from 'fresh' antitoxic serum. The second group was treated in the same way but using serum of rabbits treated with RNA-I-C from 'stored' antitoxic serum; the third and fourth groups were used as controls, using normal and hyperimmune rabbit's serum respectively.

The MRD was determined in a group of rabbits by several dilutions of toxin according to Jensen[5]. The MRD so determined was injected in a final volume of 0·1 ml. into the previously shaved abdominal skin of the rabbits treated 24 h before with RNA-I-C and, as a control, of hyperimmune rabbits.

Table 1. Effect of 'Storage' on the RNA Content of Immune Serum

RNA	Fresh serum	Stored for 40 days
mg/100 ml.	9·64 ± 0·57	5·60 ± 0·75
Per cent	100·00	58·00

Table 2. Effect on Guinea-pigs of Various Amounts of Diphtheria Toxin neutralized by Various Antitoxic Sera *in vitro*

	MLD of diphtheria toxin						
	1	2·5	5	7·5	10	15	20
Guinea-pigs treated with toxin neutralized by:							
Serum from rabbits treated with RNA-I-C from 'fresh' serum	—	—	—	—	—	—	local hyperaemia
Serum from rabbits treated with RNA-I-C from 'stored' serum	—	—	local oedema eschar	death	death	death	death
Normal rabbit serum	death	death	death	death	death	death	death
Hyperimmune rabbit serum	—	—	—	—	—	—	—

In addition, MRD neutralizing tests were made: dilutions of various antitoxic sera from 1:500 to 1:128,000 were incubated for 4 h at 37° C with the MRD of toxin and then injected, in a final volume of 0·1 ml., into the shaved abdominal skin of normal rabbits. Four groups of animals were used: to the first group was administered MRD pre-incubated with serum of rabbits treated with RNA-I-C from 'fresh' antitoxic serum, to the second the MRD pre-incubated with serum of rabbits treated with RNA-I-C from 'stored' antitoxic serum; in the third group and in the fourth the MRD pre-incubated with hyperimmune and normal rabbit serum was used.

The immune serum 'stored' for several days at +2° C undergoes a perceptible decrease of its RNA content so that, after 40 days, it is about half that of 'fresh' serum (Table 1).

RNA from 'fresh' anti-diphtheria toxin rabbit's serum, when injected into normal rabbits, elicits an antibody response which can protect guinea-pigs from the effects of the diphtheria toxin (neutralization test *in vitro*): in all the animals injected with toxin pre-incubated with serum of rabbits treated with RNA-I-C from 'fresh' antitoxic serum no paresis was found, and a hyperaemic and oedematous halo at the point of injection was observed only at the highest dose of toxin; the animals of this group were still living sixty days after treatment. Similar results were obtained in the fourth group in which antitoxic hyper-immune serum was used. All the animals of the third group, in which toxin neutralized with normal serum was used, were dead within 96 h, surviving according to the dose injected. Finally, in the guinea-pigs of the second group, in which toxin neutralized with serum of rabbits treated with RNA-I-C from 'stored' antitoxic serum was used, only those animals treated with 1, 2·5 and 5 MLD survived sixty days after treatment; the others were dead within 96 h (Table 2). Similar results were obtained using RNA-I-C from 'stored' antitoxic horse serum.

The MRD of diphtheria toxin did not induce the formation of a hyperaemic halo within 48 h in the rabbits which had received antitoxic RNA-I-C 24 h previously, or in the rabbits immunized with toxoid, used as a control. The MRD pre-incubated with normal rabbit's serum did not give a typical reaction within 48 h until serum dilutions reached 1:1,000; in the rabbits which had received the MRD neutralized with serum from animals treated with 'fresh' RNA-I-C, it was neutralized by serum diluted 1:128,000; in the rabbits treated with the MRD neutralized by serum from rabbits injected with 'stored' RNA-I-C the highest dilution able to prevent the typical hyperaemic reaction was 1:32,000 (Table 3).

As observed in our earlier work, it appears that it is possible to obtain from hyperimmune anti-diphtheria rabbit serum an RNA (RNA-I-C) that can induce a precocious anti-diphtheria response when administered by a single intravenous injection to normal rabbits. The serum of animals treated with this RNA could neutralize the diphtheria toxin *in vitro*. Storage of immune sera at +2° C affects their RNA-I-C content so that the 'stored' immune sera have a greatly decreased capacity to induce a rapid antibody response by their RNA-I-C; it is very probable that the responsibility for that is the high ribonuclease content of the serum[6]. Further investigations are in progress.

Table 3. *In vitro* Neutralization of the Diphtheria Toxin MRD in Rabbits by Various Antitoxic Sera

	Dilutions								
	1 : 500	1 : 1,000	1 : 2,000	1 : 4,000	1 : 8,000	1 : 16,000	1 : 32,000	1 : 64,000	1 : 128,000
Serum of rabbits treated with RNA-I-C from 'fresh' serum	−	−	−	−	−	−	−	−	−
Serum of rabbits treated with RNA-I-C from 'stored' serum	−	−	−	−	−	−	−	+	+
Hyperimmune rabbit serum	−	−	−	−	−	−	−	−	−
Normal rabbit serum	−	−	+	+	+	+	+	+	+

Key: −, no skin reaction within 48 h; +, 8 mm erythematous halo within 48 h.

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PATHOLOGY

Localization of Tumours and Drugs on the Chorio-allantoic Membrane of the Chick Embryo

Because the chick embryo is immunologically immature during the first two weeks of its development, heterologous tissues such as human and rat tumours can be grown on the highly vascular chorio-allantoic membrane (CAM) of this organism without the necessity of pre-treating it in order to suppress the immune response. Tumours grown in this way have been used to assess the effects of potential anti-tumour drugs[1-6].

The technique, although seemingly very simple, is not without difficulties, and some of these are sufficiently severe to make interpretation of the results, if not impossible, certainly problematical. Two difficulties in particular have prevented the wider use of this method in testing for anti-tumour drugs: the large variation in growth rate of implanted tumour fragments, which results in uncertain control values in relatively small groups of eggs, and the sharp increase in operative mortality when successive doses of the drug under test are administered into the yolk sac or intravenously, which are the usual routes of drug administration in this system.

We sought to overcome these disadvantages by applying disks of the kind shown in Fig. 1 to the CAM and filling the six peripheral compartments with tumour cells, and the larger central well with the drug to be examined.

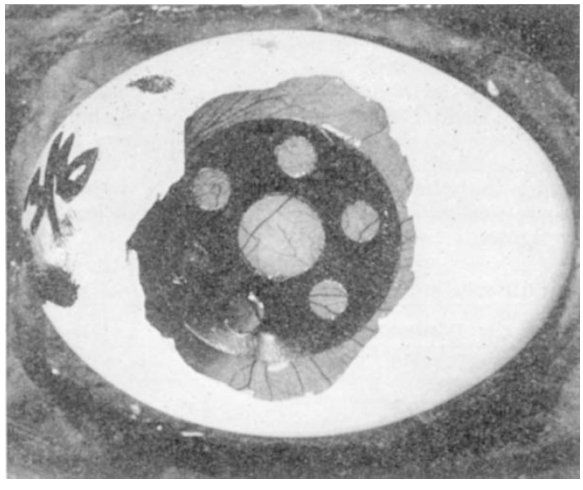

Fig. 1. Disk in place on the vascular CAM. The window in the shell is much enlarged in order to show the whole of the disk in position

Our aim was twofold; first, if an approximately equal number of tumour cells were to fill the compartments of the disks, then it seemed possible that tumour nodules of approximately equal size might develop. Depression of size or weight of the nodules by a drug would then be amenable to relatively simple assessment. If, on the other hand, the tumours were found to be of different sizes, then because six tumours instead of the usual one could be grown on one CAM, statistical evaluation again of size, weight, or possibly number of 'takes' might be on somewhat firmer ground.

A second aim was to be able to give drugs in a technically simple and consistent way without at the same time applying them directly to the tumour cells, as might be done in tissue culture. It will be seen that the method we describe goes some way towards achieving these aims. (An outline of this work was given to the British Pharmacological Society, Bristol, 1964, ref. 7.)

Fertilized White Leghorn eggs were incubated at 103° F and implantation was carried out on the 8th day of incubation. The method of approaching and dropping the CAM was essentially that of Ballantyne[6].

Disks were placed on to the 'dropped' CAM through the window made by removing a portion of the shell and the underlying shell membrane. They adhered closely to the CAM, the holes in the disks forming six tight outer compartments and a central well, all having the vascular CAM as their base. The disks were cut from black polythene sheet 0·0125 mm thick and each weighed 40 mg. Before use they were heat sterilized between sheets of filter paper to prevent them from curling. The disks were used once only.

In order to see how watertight the compartments were when the disks were in position on the CAM, methylene blue was dropped into them in one experiment and eosin in another. Any tendency for liquids to run together under the disks could then be followed with the naked eye when methylene blue was used, or under ultra-violet light in the case of eosin.

In the present experiments, only the Walker 256 rat carcinosarcoma in the form of a suspension of cells was used, though in another series of experiments to be reported later, other tumours were also examined.

The inoculated cells were derived from either the solid or ascitic forms of the tumour. Cells from the solid Walker tumour were packed by centrifugation and administered either in this highly concentrated form (100 per cent packed cells) or as a 10 per cent cell suspension in sterile saline. Cells from the ascitic tumour were concentrated in a similar manner; the dense upper layer of the cell pellet being withdrawn into a 0·25-ml. syringe and used directly for inoculation (100 per cent ascites cells). All suspensions were applied dropwise through a 26-gauge needle to each of the six compartments in rotation, until the total volume had been given. This varied from 0·003 to 0·03 ml. according to the cell concentration. After inoculation of the cells the eggs were sealed with 'Sellotape' and returned to the incubator.

Solutions of drugs used, in the concentration indicated in Table 1, were applied to the central well at intervals between 0 and 96 h after tumour inoculation through a small temporary slit in the 'Sellotape' seal. Drugs used were *bis*(2-chloroethyl) methylamine hydrochloride (mustine hydrochloride; HN2) and *cyclo*phosphamide. They were given in a total volume of 0·05 ml. per egg. All drugs were given as a single dose except in the last experiment (see Table 1).

Eggs were inspected daily and embryos that had died before the 6th day of incubation were discarded. At that time all the embryos were killed. A segment of the CAM sufficiently large to include the disk and about 5 mm of the surrounding membrane was cut out and placed in neutral buffered formalin. The fixed membranes were then examined for the presence of tumour in the six compartments. Tumour growth in any one compartment