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The Effect of Various Immunosuppressive Agents on Mouse Peritoneal Macrophages and on the *in vitro* Phagocytosis of *Escherichia coli* O4:K3:H5 and Degradation of ^{125}I-labelled HSA–Antibody Complexes by these Cells

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Summary. Large doses of hydrocortisone, cyclophosphamide, and methotrexate injected subcutaneously, and whole-body irradiation (500 rads) caused a reduction in the number of peritoneal cells (PE cells) obtained after intraperitoneal injection of the treated mice with proteose–peptone. The same dose of cyclophosphamide and irradiation induced morphological changes in PE macrophages. There were more giant cells in the peritoneal exudates from treated mice as compared to control mice.

'Pharmacological' and larger doses of hydrocortisone, methotrexate and azathioprine or anti-lymphocyte globulin had no effect on the *in vitro* phagocytic capacity of proteose–peptone-stimulated mouse PE macrophages. This also applied to doses of up to 50 mg/kg of cyclophosphamide. In contrast, whole-body irradiation (500 rad) and 100 mg/kg of cyclophosphamide decreased the phagocytic capacity of mouse macrophages *in vitro* and reduced the ability of PE cells to degrade ^{125}I-labelled HSA–antibody complexes *in vitro*. The greatest effect was noted 4–5 days after whole-body irradiation or four to five subcutaneous injections of cyclophosphamide.

INTRODUCTION

It is a well-known fact that patients treated with immunosuppressive agents show a decreased resistance to infection, which could partly be due to impaired phagocytosis.

There are various controversial reports in the literature on the effect of immunosuppressive agents, notably hydrocortisone and irradiation, on the phagocytic ability of macrophages *in vivo* and *in vitro* (reviewed by Berenbaum, 1967). These discrepancies could be due to the different experimental methods used to evaluate phagocytosis. In order to avoid such a pitfall, we have used as standardized a procedure as possible to study some of the immunosuppressive agents in common use.

Mice were used as experimental animals, injected subcutaneously with the immuno-

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suppressive agents. Their peritoneal macrophages were tested, *in vitro*, for their ability to phagocytose *E. coli* and degrade radioactive antigen–antibody complexes.

MATERIALS AND METHODS

Animals

Inbred C3H mice of both sexes, bred at the Statens Seruminstitut, were used. The mice were 3–5 months old, average weight about 25 g.

Antigens

Human serum albumin (HSA) was prepared at the Statens Seruminstitut.

Formalin-killed capsular strain of *E. coli*, U4/41, serotype O4:K3:H5, was kindly provided by Dr Ida Ørskov, International *Escherichia* Centre (WHO). The bacteria were adjusted to a concentration of $1{\cdot}0 \times 10^9$ bacteria per millilitre, and opsonized with rabbit O antiserum against *E. coli* O antigen before use (anti-O4, Statens Seruminstitut). Eight different *E. coli* batches were used for the experiments.

Antiserum

Pool 82 prepared by injecting rabbits twice subcutaneously (s.c.) with 0·1 mg of HSA in 0·5 ml of saline and 0·5 ml of Freund's incomplete adjuvant with an interval of 1 month between injections. Only sera with high titres of precipitating antibodies were used for the pool.

Iodination

HSA was labelled with carrier-free ^{125}I according to the method of Hunter and Greenwood (1962), at a specific activity of about 0·2 Ci/μg. The carrier-free ^{125}I was obtained from the Radiochemical Centre, Amersham, Bucks.

Treatment of mice

All mice were injected intraperitoneally with 2 ml of sterile 10 per cent proteose–peptone (Difco) in saline 3 days prior to harvesting peritoneal exudate cells (PE cells).

For the majority of the experiments the immunosuppressive drugs were injected daily, s.c., for 5 days. The experiments were carried out on the 6th day. All doses (except for anti-lymphocyte globulin) were in 1 ml. The same dose in mg/kg was given to each mouse, assuming a weight of 25 g per mouse. The amounts injected into six mice per group (phagocytosis) and twelve mice per group (degradation) were as follows.

Cyclophosphamide (Endoxan® Astra): 1, 10, 50 and 100 mg/kg/day dissolved in sterile-buffered saline (PBS) (pH 7·2, s.c., ×5). 100 mg/kg of cyclophosphamide was also injected s.c. into groups of six mice (or twelve) and the experiment was carried out on the day following the second, third, fourth, fifth (sixth) injection. Controls: *PBS* (1 ml, s.c., ×5).

Methotrexate (Methotrexate® Lederle): 0·1, 1, 10 and 25 mg/kg/day dissolved in PBS (s.c., ×5). Controls: PBS (1 ml, s.c., ×5).

Azathioprine sodium salt (Imurel® Burroughs-Wellcome): 1, 10, 50 and 100 mg/kg/day dissolved in saline (s.c., ×5). Controls: saline, pH adjusted to 5·9, 7·5, 9·5 and 9·5, respectively.

Sodium hydrocortisone succinate (Solu-Cortef® Upjohn, Mix-o-vial): 0·5, 5, 50 and 500 mg/kg/day dissolved in accompanying solute and then diluted with PBS (s.c., ×5).

Controls: PBS (1 ml, s.c., ×5).

Rabbit anti-mouse (C3H) lymphocyte globulin. Kindly supplied by Dr Claus Lundstedt, Institute for Medical Microbiology, Copenhagen. Haemagglutination titre, 1:4, leuco-agglutination titre, 1:16 (0·25 ml, s.c., ×5). Controls: normal rabbit globulin (NRG) (0·25 ml, s.c., ×5). Also supplied by Dr Claus Lundstedt.

Whole-body X-irradiation. This was carried out at the Radiophysical Laboratory, Radium Station, with a Siemens Stabilipan X-ray apparatus. Dose rate 24 rads/min. Irradiation factors were 200 kV, 16 mA, filter 0·5 mm Cu half value layer (HVL) 1·1 mm Cu, target distance 66 cm. 50, 100, 300 and 500 rads were given once and experiments were carried out 5 days after the irradiation. In addition, mice were irradiated with 500 rads and these experiments were carried out on days 2, 3, 4, 5 and 6 after irradiation. Controls: sham-irradiated, by placing in the wooden container for the same time interval.

The only mice which showed evidence of side effects other than changes in body weight were those treated with methotrexate, 10 mg/kg, s.c. or more daily for 5 days, which had diarrhoea and disorderly hair. After 25 mg/kg the mice were lethargic. Mice given hydrocortisone, 500 mg/kg/day, showed an increase in weight, whereas mice treated with large doses of cyclophosphamide and methotrexate and X-irradiation showed a loss in weight.

Preparation of PE cells

Mice were killed by cervical dislocation and cells were harvested by washing out the peritoneum with 3 ml of saline containing 15 i.u. of heparin and 5 per cent normal rabbit serum (NRS).

(a) *For phagocytosis.* The PE cells from each group of mice were pooled, washed twice at 700 ***g*** for 3 minutes and resuspended in 5 ml of Gey's solution containing 20 per cent NRS, and finally the cell suspension was adjusted to $5{\cdot}0 \times 10^6$ per ml where possible.

(b) *For degradation.* Exudate cells from pairs of mice were pooled giving a total of six batches of cells for each group. The cells were washed twice with Gey's solution for 3 minutes at 700 ***g*** and resuspended in Gey's solution to give 2×10^6 cells per 0·1 ml.

A smear was made of all the cell suspensions using a spreading device (Doré and Balfour, 1965). Differential cell counts were performed on these smears, 400 cells being counted.

For the purposes of this article macrophages were denoted as large mononuclear cells, cytoplasm/nucleus ratio about 1, and giant cells as large bi- or multi-nuclear cells having the morphology of macrophages.

Viable counts. All cells harvested *in vivo* were tested for viability using trypan blue. 0·1–0·2 ml of cell suspension was mixed with 0·2–0·4 ml of an 0·2 per cent solution of trypan blue in saline. The number of damaged cells was counted 2–3 minutes after mixing. Ninety-four to ninety-six per cent of the cells excluded dye. Cells used *in vitro* were tested after the experiment and 80–90 per cent excluded dye. Since only short staining times were used, the test was carried out strictly to the times stated because it has been pointed out that the percentage of stained cells depends on the time between adding the stain and performing the count (Black and Berenbaum, 1964).

Procedure for phagocytosis of E. coli

0·5 ml of cell suspension ($2{\cdot}0–5{\cdot}0 \times 10^6$ macrophages per millilitre), containing 20 per cent NRS was incubated with 0·5 ml bacterial suspension ($2{\cdot}5–5{\cdot}0 \times 10^8$ per millilitre) in sterile siliconized test tubes (macrophage:bacteria ratio 1:50–1:165). The mixture

was incubated at 37° for 15 minutes in a rotator. Immediately after incubation the tubes were placed on ice. The mixture was centrifuged at 470 *g* for 3 minutes and the cells were then washed twice with Gey's solution containing 5 per cent NRS. After the final wash the cells were suspended in 1–3 ml of cold sterile saline. Smears were made of each cell suspension as mentioned above and stained with Wright's stain. A total of 400 macrophages was counted; bacteria were considered to be phagocytosed if they were clearly within the membrane, and adhering or trapped bacteria were not included. The number of bacteria in each macrophage, up to a maximum of twenty, was counted. These results are expressed in two ways: (a) the number of bacteria phagocytosed per macrophage; and (b) the number of macrophages containing a given number of bacteria.

Procedure for the degradation of ^{125}I-labelled HSA–antibody complexes (according to Sorkin and Boyden, 1959)

As mentioned above, six batches of treated and control cells were prepared and adjusted to 2×10^6 cells per 0·1 ml. As far as possible the degradation procedure was carried out in triplicate.

To 0·1 ml cells was added 0·8 ml Gey's solution, 0·1 ml antiserum number 82 and 10 μg/20 μl ^{125}I-labelled HSA. Six appropriate controls without cells were also prepared. The radioactivity in each tube was first measured and then the mixtures were incubated at 37° for 3 hours.

The cells and antigen–antibody complex were centrifuged at 700 *g* and the supernatant removed. 0·2 ml of 25 per cent trichloracetic acid was added, the mixture left to stand for 5 minutes, centrifuged at 970 *g* for 10 minutes and the radioactivity in the supernatant measured. Evaluation of the breakdown of ^{125}I-labelled HSA–antibody complexes is based on the non-protein-bound radioactivity in the supernatant as compared to the original radioactivity added. Radioactivity was measured in a well-type scintillation counter.

Statistical evaluation

Phagocytosis experiment. (a) The cell concentration was calculated from the cell counts in the resuspending volume, taking into consideration the original volume harvested from the peritoneum. These cell concentrations were compared by assuming a Poisson distribution of the cell counts. Approximate *u* values (standard normal deviates were calculated on the basis of this assumption.

(b) The phagocytosis counts were compared using Wilcoxon's two sample test. The macrophages were divided into groups according to the number of bacteria ingested (0, 1–2, 3–5, 6–10, 11–15, 16–20, >20). The result is expressed as a *u* value (Mann-Whitney *u* test).

RESULTS

TOTAL CELL CONCENTRATION

The effect of treatment with immunosuppressive agents on the total PE cell concentration is illustrated in Table 1. It is clear that treatment for 5 days s.c. with various doses of hydrocortisone, cyclophosphamide, methotrexate, azathioprine and a single dose of 300–500 rads caused a significant decrease in the total PE cell concentration. Small doses of the immunosuppressive drugs had no effect on the cell concentration.

The total PE cell concentration (not shown here) was also estimated for the degradation

TABLE 1

EFFECT OF IMMUNOSUPPRESSIVE AGENTS ON THE TOTAL PE CELL CONCENTRATION OF 'POOLS' FROM FIVE TO SIX MICE INJECTED i.p. 3 DAYS PREVIOUSLY WITH 10 PER CENT PROTEOSE–PEPTONE

Immunosuppressive agent	Dose (mg/kg/day)	Cells/ml	
		Treated	Control
Hydrocortisone succinate (s.c., ×5)	5	$3{\cdot}8\times10^6$	$5{\cdot}2\times10^6$*
	50	$2{\cdot}8\times10^6$	$4{\cdot}9\times10^6$*
	500	$1{\cdot}9\times10^6$	$4{\cdot}2\times10^6$*
Cyclophosphamide (s.c., ×5)	10	$4{\cdot}0\times10^6$	$5{\cdot}7\times10^6$*
	50	$1{\cdot}1\times10^6$	$4{\cdot}5\times10^6$*
	100	$5{\cdot}4\times10^6$	$4{\cdot}2\times10^6$*
Methotrexate (s.c., ×5)	1	$4{\cdot}5\times10^6$	$6{\cdot}1\times10^6$*
	10	$4{\cdot}3\times10^6$	$5{\cdot}0\times10^6$*
	25	$2{\cdot}8\times10^6$	$4{\cdot}7\times10^6$*
Azathioprine (s.c., ×5)	1	$4{\cdot}2\times10^6$	$5{\cdot}5\times10^6$*
	10	$3{\cdot}5\times10^6$	$3{\cdot}8\times10^6$
	50	$4{\cdot}2\times10^6$	$4{\cdot}9\times10^6$*
	100	$3{\cdot}8\times10^6$	$5{\cdot}3\times10^6$*
Whole-body irradiation (×1)	(rads)		
	300	$1{\cdot}6\times10^6$	$4{\cdot}1\times10^6$*
	500	$9{\cdot}8\times10^5$	$4{\cdot}8\times10^6$*

* Statistically significant difference between treated and control animals. ($P<5$ per cent.)

experiments in which mice were treated with cyclophosphamide s.c. for 2–6 days, or whole-body irradiation (500 rads) and then killed at 2–6 days after irradiation. These cell counts were also reduced. The lowest cell concentration was attained after four daily doses of cyclophosphamide and 4 days after whole-body irradiation (500 rads). The value for the treated animals was from $1–2\times10^6$ cells per mouse, which remained constant for up to 6 days after treatment. The control values varied from $8–12\times10^6$ cells per mouse.

DIFFERENTIAL COUNTS AND MORPHOLOGY

The percentage of macrophages in all control and treated groups was about 67–87 per cent, the majority being between 70 and 80 per cent for both sets of groups. Other cells comprised from 10–20 per cent mononuclear cells including lymphocytes, 5–10 per cent eosinophils and 1–7 per cent giant cells.

About 7 per cent giant cells were found in the group treated with 100 mg/kg of cyclophosphamide s.c., ×5 and 3 per cent with 50 mg/kg of cyclophosphamide s.c., ×5. The group treated with 500 rads had between 2–3 per cent giant cells. In all the other groups, both control and treated, the percentage of giant cells was from <1 to 1 per cent. In both the 100 mg/kg cyclophosphamide-treated and the irradiated groups the numbers of giant cells reached a maximum on the 3rd to 4th day of treatment, thereafter remaining constant up to 6 days of treatment.

The cells from mice treated with 50 and 100 mg/kg of cyclophosphamide and 500 and 300 rads were greatly altered morphologically. The majority of the macrophages were of a larger diameter than usual and were heavily vacuolated (compare Fig. 1 with Fig. 2).

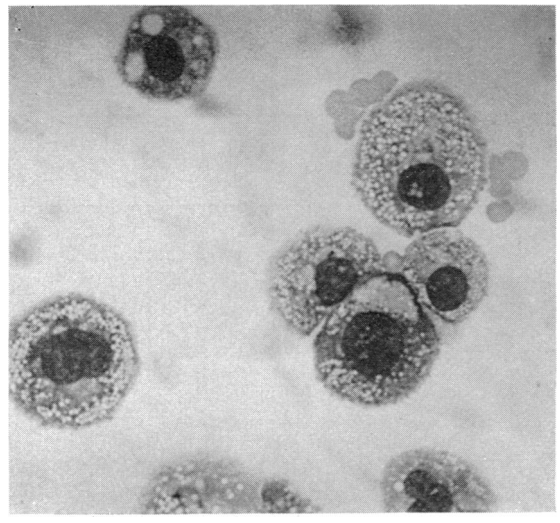

FIG. 1. Peritoneal macrophages from mice treated with 100 mg/kg of cyclophosphamide s.c. for 5 days. Note the heavy vacuolization. (Magnification ×320.)

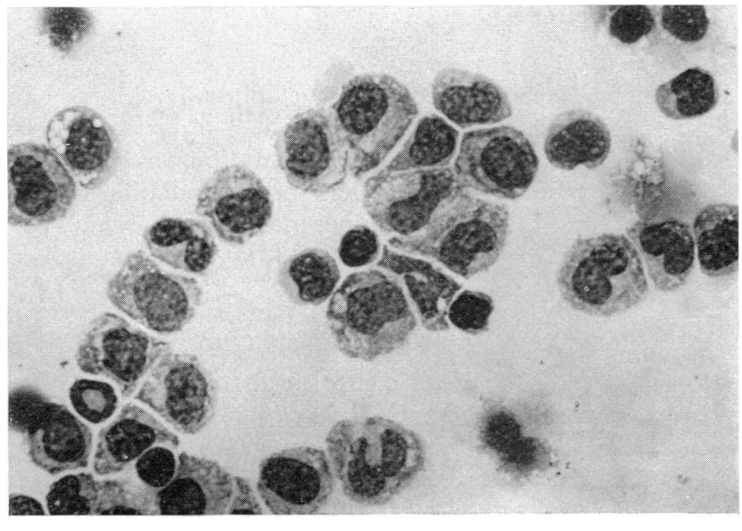

FIG. 2. Peritoneal macrophages from untreated mice. (Magnification ×320.)

PHAGOCYTOSIS OF *E. coli*

After various doses of immunosuppressive agents s.c. for 5 days

Only two of the immunosuppressive agents, namely cyclophosphamide (100 mg/kg) and X-irradiation (500 rads), caused a significant decrease in phagocytosis (see Figs 3 and 4). The column which represents macrophages with no bacteria is not drawn to scale, but the figure on top indicates the total number of macrophages.

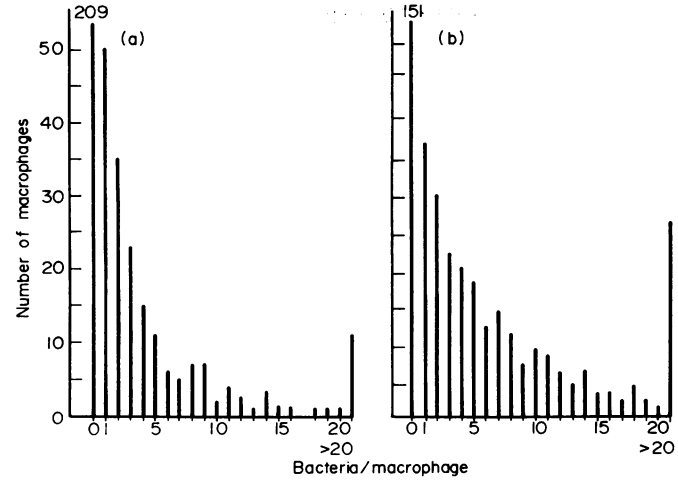

FIG. 3. This represents the number of PE macrophages that have phagocytosed a given number of bacteria (from 0 to >20) *in vitro*. The first column illustrates the number of macrophages that have not ingested bacteria. The treated cells were from mice injected with 100 mg/kg of cyclophosphamide s.c. for 5 days. (a) Treated. (b) Control.

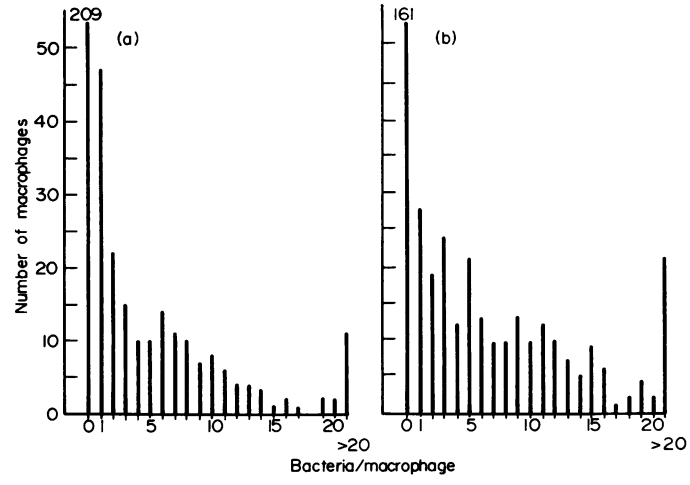

FIG. 4. This represents the number of PE macrophages that have phagocytosed a number of bacteria (from 0 to >20) *in vitro*. The first column illustrates the number of macrophages that have not ingested bacteria. The treated cells were from mice which had received 500 rads whole-body irradiation 5 days prior to the experiment. (a) Treated. (b) Control.

After cyclophosphamide 100 *mg/kg and* 500 *rads for various time intervals*

PE cells from mice which were treated with 100 mg/kg cyclophosphamide s.c. for 2–5 days and cells from mice given one dose of 500 rads and then killed 2–5 days after irradiation were also tested for their ability to phagocytose *E. coli*.

Table 2 illustrates the phagocytosis of *E. coli* 2, 3, 4 and 5 days after treatment with cyclophosphamide. It is quite clear that fewer macrophages ingest large numbers of bacteria as compared to control cells, and that a larger proportion of treated cells do not phagocytose at all, from 3 to 5 days after treatment. This result is highly significant.

PE cells from irradiated mice also showed markedly reduced phagocytosis of *E. coli*

TABLE 2

PHAGOCYTIC ABILITY OF MACROPHAGES FROM MICE TREATED WITH CYCLOPHOSPHAMIDE (100 mg/kg)

Number of days injected	Cyclophosphamide (100 mg/kg)	Number of macrophages (bacteria/macrophage) 0	1–2	3–5	6–10	11–15	16–20	>20	u Value*
2	Treated	159	33	32	56	28	18	65	−0·25
2	Control	153	50	43	33	26	29	56	
3	Treated	157	39	25	46	45	24	62	2·21
3	Control	135	38	24	48	32	23	96	
4	Treated	201	73	44	39	23	8	11	7·36
4	Control	153	21	17	31	39	21	112	
5	Treated	209	85	49	27	11	4	11	5·32
5	Control	151	67	60	53	27	12	26	

* If u is numerically greater than 2, then the difference is significant at the 5 per cent level. The calculation of the u value is based on Wilcoxon's two-sample test.

3 days after irradiation (see Table 3). Here again it is obvious that less bacteria are phagocytosed as compared to the controls. It should be noted that only control and treated for each day should be compared due to the variation in the phagocytosis of the different batches of *E. coli*.

TABLE 3

PHAGOCYTIC ABILITY OF MACROPHAGES FROM MICE TREATED WITH X-IRRADIATION (500 rads)

Days after treatment	X-irradiation (500 rads)	Number of macrophages (bacteria/macrophage) 0	1–2	3–5	6–10	11–15	16–20	>20	u Value
2	Treated	107	45	51	74	40	29	47	2·68
2	Control	115	28	24	60	53	41	81	
3	Treated	133	54	56	57	30	17	52	4·50
3	Control	107	49	26	31	39	27	114	
4	Treated	167	51	49	51	34	12	35	4·25
4	Control	128	29	49	59	35	22	71	
5	Treated	209	69	35	50	18	7	11	4·27
5	Control	161	47	57	53	43	15	21	

* If u is numerically greater than 2, then the difference is significant at the 5 per cent level. The calculation of the u value is based on Wilcoxon's two-sample test.

DEGRADATION OF ^{125}I-LABELLED HSA–ANTIBODY COMPLEXES

Of the immunosuppressive agents tested (s.c., ×5), hydrocortisone succinate and anti-lymphocyte globulin had no significant effect on degradation. On the other hand, methotrexate and azathioprine caused a significant increase in degradation (Table 4). In contrast, cyclophosphamide and whole-body irradiation caused a significant decrease in the degradation of ^{125}I-labelled HSA–antibody complexes as compared to the controls (Table 4). Since it was of interest to know when the greatest effect occurred the degradation of the radioactive complexes was investigated further at different time intervals after treatment with cyclophosphamide and after irradiation.

Fig. 5 illustrates the course of degradation of the radioactive complexes by PE cells obtained after injection of cyclophosphamide s.c. The control values were constant for the

TABLE 4

PERCENTAGE DEGRADATION OF ^{125}I-LABELLED HSA–ANTIBODY COMPLEXES BY MOUSE PE CELLS, AFTER TREATMENT WITH IMMUNOSUPPRESSIVE AGENTS

Agent*		Percentage degradation	Average value	u‡ Value
Azathioprine (100 mg/kg)	Control†	11·3, 12·2, 9·0, 9·7, 9·3, 9·0	10·1	−3·08
	Test	13·4, 14·6, 14·1, 14·4, 13·9, 9·5	13·3	
Cyclophosphamide (100 mg/kg)	Control†	11·1, 10·8, 7·2, 11·9, 10·6, 11·9	10·6	6·06
	Test	8·2, 1·4, 3·5, 1·6, 3·7, 6·9	4·2	
Methotrexate (10 mg/kg)	Control†	8·6, 9·4, 10·6, 12·3, 11·2, 11·5	10·6	−4·73
	Test	15·2, 13·4, 13·9, 15·7, 18·4, 16·8	15·6	
X-irradiation (500 rads)	Control†	9·9, 11·7, 10·8, 11·0, 11·2, 10·0	10·8	4·76
	Test	4·4, 5·7, 5·7, 3·0,6·6, 9·2	5·8	

* All groups received five daily doses of the immunosuppressive agents, except for whole-body irradiation, which was given once. Experiments were carried out on the 6th day, for X-irradiation 5 days after irradiation.

† Each figure in the control series is the mean of triplicate determinations, and in the test column either duplicate or triplicate determinations.

‡ The calculation of the u value is based on Wilcoxon's two-sample test.

different time intervals. By day 3 treated cells degraded a higher percentage of the complexes than control cells, but the percentage degradation fell rapidly to less than the control value after four injections of cyclophosphamide. This value decreased again after five injections and then increased after the 6th injection but did not reach the control value.

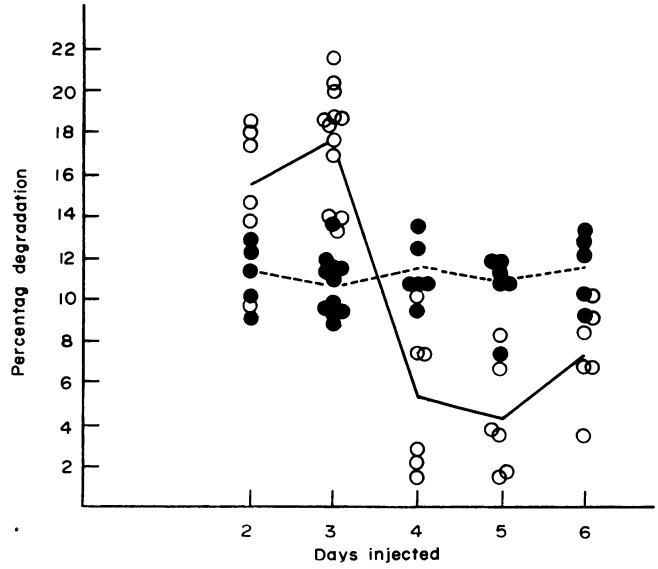

FIG. 5. Percentage degradation of ^{125}I-labelled HSA–antibody complexes by PE mouse macrophages *in vitro* at various time intervals after injection. Each point represents a duplicate or triplicate determination for each mouse (see text). (●– –●) Control. (○—○) Mice treated with 100 mg/kg of cyclophosphamide s.c. each.

After whole-body irradiation (Fig. 6), the PE cells of the treated mice did not differ from the untreated PE cells in their ability to degrade the complexes until the 4th day after irradiation. On day 4 the percentage degradation was significantly decreased as

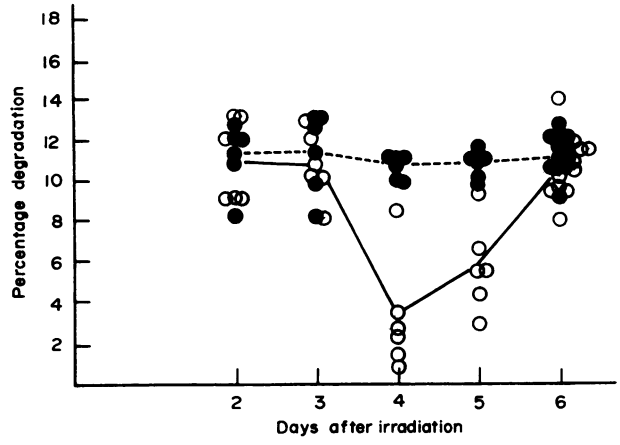

FIG. 6. Percentage degradation of ^{125}I-labelled HSA–antibody complexes by PE mouse macrophages *in vitro* after a single whole-body irradiation of 500 rads. Each point represents a duplicate or triplicate determination for each mouse (see text). (●– –●) Control. (○—○) Mice irradiated with 500 rads each.

compared to the control value. The percentage degradation increased again on the 5th day and on the 6th day reached the value for control cells.

DISCUSSION

Furth and Cohn (1968) demonstrated that PE macrophages arise from rapidly proliferating precursor cells in the bone marrow, which mature in the blood stream, and from there reach the peritoneum. The reduction in the cell count in the peritoneal cavity caused by cyclophosphamide, methotrexate and irradiation, which has been observed in the present experiments, could be explained by the antiproliferative effect of these agents (Calabresi and Parks, 1970).

Although the number of cells in the peritoneal cavity was reduced after exposure to cyclophosphamide, methotrexate and irradiation, the percentage of macrophages in the exudates remained essentially unaltered. This is in agreement with the experiments of Kornfeld and Greenman (1966), although they used unstimulated mice, whereas ours were stimulated. However, others have shown that hydrocortisone (Thompson and Furth, 1970) and X-rays (Nelson and Becker, 1959) reduced the peritoneal exudate in mice after prior stimulation.

It is a well-known fact that alkylating agents and X-rays can cause the formation of giant cells (Puck and Marcus, 1956; Brewer, Comstack and Aronow, 1961). Such changes have also been observed in bone marrow cells after cyclophosphamide treatment (Castaldi, Zavachi, Fiocchi and Trotta, 1970). We have observed that apart from the increase in size of the PE macrophages, resulting finally in giant cells, there is also an increase in the number of vacuoles (see Fig. 1). This was not observed by Castaldi *et al.* (1970), although they used doses of cyclophosphamide which exceeded those used in the present experiments. The difference in results could well be due to the fact that we used stimulated macrophages.

Large doses of cyclophosphamide and 500 rads reduced the capacity of PE macrophages to phagocytose *E. coli*, the greatest effect being seen after three to four injections (cyclo-

phosphamide) or 3–4 days after irradiation (Tables 2 and 3). Although cells from similarly treated mice were incapable of degrading antigen–antibody complexes to the same extent as control cells, this effect on degradation was only transient (Figs 5 and 6), whereas the effect on phagocytosis persisted (Tables 2 and 3).

A point of interest is that after two to three injections with cyclophosphamide the PE cells degraded significantly more radioactive antigen–antibody complex than untreated PE cells. This suggests that cyclophosphamide, initially, had a stimulatory effect on the enzymes of the PE cells despite the fact that the phagocytic ability of the cells was diminished.

Methotrexate and azathioprine also enhanced the intracellular digestion of the complexes (Table 4). Here again, this may indicate that macrophages from mice treated with these agents may be stimulated enzymatically even though the drugs have no effect on phagocytosis in these short-term experiments. An explanation for these results could be that some immunosuppressive agents can have an effect on both phagocytosis and enzyme production and that this does not necessarily occur simultaneously.

It was observed in our experiments that intracellular digestion was unaffected by hydrocortisone, corresponding to its lack of effect on phagocytosis. This is in contrast to the results of Wiener, Marmary and Curelaru (1972), which indicated that hydrocortisone in high concentration *in vitro* in cultures of macrophages lowered the phagocytosis of particles but lacked effect on their degradation. These two types of experiments serve to illustrate the difference in results obtained when cells are treated *in vivo* or *in vitro* with an immunosuppressive agent, as in both cases bacteria were employed for phagocytosis and isotopically labelled particles to test for intracellular digestion.

Despite the fact that our experimental conditions were standardized as far as possible, we are aware that such *in vitro* phagocytosis experiments can be affected by many different factors. This is exemplified by the variation in phagocytosis of the different batches of bacteria, which may be due to changes in the antigenic composition on the surface of the *E. coli* bacteria. Nevertheless, the combination of *in vivo* and *in vitro* experiments may well offer certain advantages over a purely *in vitro* experimental procedure, since the immunosuppressive agents were administered under more physiological conditions, and particularly since the results with *in vitro* techniques have been very contradictory (for review, see Berenbaum, 1967).

The actual mechanism by which cyclophosphamide and irradiation can inhibit phagocytosis is not known. However, these agents are antiproliferative (Calabresi and Parks, 1970) and could inhibit the formation of precursor cells in the bone marrow and reduce the number of monocytes in the peripheral blood from which PE macrophages are recruited (Furth and Cohn, 1968). After treatment with antiproliferative agents, an injection of proteose–peptone was incapable of inducing a new influx of cells from the peripheral blood into the peritoneal exudate, although this is what normally occurs, suggesting that precursor cells are not being formed in the bone marrow. It is, therefore, conceivable that the majority of the cells which remain in the peritoneal exudate are the 'old' cells which were present at the time of administration of the immunosuppressive agent.

Cyclophosphamide and irradiation may well prevent the accumulation of 'new' cells from the bone marrow, but these agents might also kill a proportion of the 'old' cells in the peritoneum. What would remain would be survivors which might be committed to dealing with the debris present, and thus they would seem to have a reduced phagocytic capacity when tested with *E. coli*. Unpublished electron microscope studies suggest that

the macrophages and giant cells from mice treated with these two agents do contain more debris than is normally seen in untreated cells. In addition, the treated cells do have a reduced phagocytic capacity for *E. coli*. This raises the possibility that there may be a difference in the phagocytic ability between 'old' and 'new' cells. This point is being investigated.

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