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The Effect of Cyclophosphamide on the Allergic Contact Reaction in Guinea Pig: Dose Effects and Influence on Peripheral Blood

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Single intraperitoneal injections of cyclophosphamide were administered 6 days before testing guinea pigs sensitized to oxazolone in order to study the effects on inflammatory cell populations in blood and dermis. Skin tests were assessed macroscopically (erythema and oedema) and microscopically (counting of the dermal inflammatory cell infiltrate). At the highest dose (300 mg/kg) the allergic contact reaction was augmented with increases in erythema and oedema and the mononuclear dermal infiltrate. At the lowest dose (75 mg/kg), redness and oedema and all components of the dermal inflammatory cell infiltrate decreased. Total and differential white blood counts up to 20 days after administration of cyclophosphamide showed that a dose-dependant leukopenia maximal around 6 days occurred. During the leukopenia the differential count showed a lymphocytosis with a marked granulocyte depletion. The augmentation of the contact allergic reaction produced at the highest dose of cyclophosphamide occurs despite a marked peripheral blood leukopenia. Cyclophosphamide's effects at the lower dose would appear to be of a non-specific anti-inflammatory nature. *Key words: Immunosuppressant; Cytostatic; Dermal cellular infiltrate; Differential blood count; Oxazolone.* (Received February 16, 1985.)

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Cyclophosphamide is a cytostatic agent which is widely used clinically for its immunosuppressant effects (for review see ref. 1). It is still, however, unclear whether these effects, especially in vivo, represent "specific" interference in immunological mechanisms or more "non-specific" anti-inflammatory effects at a local or systemic level. In this regard, the drug's differential effects on inflammatory cell populations in both blood and tissue are of importance. In experimental animal models of the contact allergic reaction cyclophosphamide can block sensitization when administered from 3 days prior to sensitization to 4 days after sensitization (2, 3, 4). Single doses prior to sensitization can augment the reaction (5). When cyclophosphamide is administered in relation to the elicitation (efferent) limb of the contact allergic reaction, we have previously seen that effects vary depending on how long prior to test the substance is administered (6). A dose of 300 mg/kg administered on the first test day, three days and six days prior to first test day, caused the macroscopic appearance and the dermal mononuclear infiltrate of tests to vary in a biphasic and independant fashion. The effect on basophil and eosinophil dermal infiltrates is monophasic with decreases at all points. The 300 mg/kg dose of cyclophosphamide, an exceptionally high but none-the-less commonly used dosage (2), is about the highest compatible with animal survival.

The aim of this study was to further investigate the cellular aspects of the effects of smaller doses of cyclophosphamide on the efferent limb of the contact allergic reaction to oxazolone, administered at a point where the 300 mg/kg dose had shown marked effects. Since the recruitment of inflammatory cells from peripheral blood to the dermal infiltrate is

a question of prime importance in any discussion of the effects of cyclophosphamide, we have also complemented previously obtained data on dose effects of the drug on peripheral blood.

MATERIALS AND METHODS

Animals. Female albino Dunkin Hartley guinea pigs weighing about 300 g at the time of sensitization were kept under standardized conditions. Prior to sensitization and testing, appropriate sites were shaved with an electric razor.

Sensitization and testing. 20 µl of 20% oxazolone in acetone was painted on a 1 cm^2 area on each side of the neck on 2 consecutive days. At test 10 µl of a 0.2% oxazolone in acetone solution was applied to 1 cm^2 areas on the flanks.

Macroscopic assessment. Prior to sacrifice, the reactions are assessed visually and palpably (0=no change or uncertain reaction, + = redness, ++ = redness with slight palpable induration and +++ = redness and obvious swelling). A "macroscopic score" for the group is obtained by dividing the total number of "+" for the group by the number of animals in the group.

Histology. After sacrifice, punch biopsies of the test site and normal skin are taken, fixed in 10% neutral phosphate buffered formalin, embedded in glycol methacrylate and polyethylene glycol (Sorvall Embedding Medium, Du Pont USA), cut in 3 µ sections and stained with May-Grünwald-Giemsa. Microscopic assessment is performed with a 1000 × oil immersion lens.

Counting. 20 fields just below the dermo-epidermal junction are counted and results presented as the average per high power field. Granulocytes are differentiated on morphological grounds into basophils, eosinophils and neutrophils. At light microscopy without special staining, mononuclear cells (lymphocytes, monocytes) are counted as a single group. Mast cells are also counted. All cell types are included in a "total" cell count. In every animal the dermal cellular infiltrate in normal skin is counted. This figure is subsequently substracted from the test area counts, giving a "cell response" (test count minus normal count) for every animal and cell type.

Statistical analysis. Mean cell responses for groups are analysed by paired T test against values for control groups of animals. Although strict statistical analysis of changes in the macroscopic score is not possible, we designate changes in the mean score as "slight" (change by 0.3–0.4), "clear" (change by 0.5–0.9) or "marked" (change by $\geq$1.0).

Peripheral blood counts. A total white blood cell count was performed on capillary blood samples using a Bürkers chamber. A differential count on a thin blood smear stained with May-Grünwald-Giemsa was also performed providing leukopenia was not marked, in which case only a mononuclear/polymorphonuclear differential was performed.

Experimental design. Cyclophosphamide was administered intraperitoneally to groups of 5 animals 6 days prior to first test day in a dose of 150 mg/kg and 75 mg/kg. Tests were applied 3, 2 and 1 days prior to sacrifice to give a 72, 48 and 24 hour reaction. The results are thus directly comparable to those previously obtained for the highest (300 mg/kg) dose (6). Control groups (n=5–10) were from the same season as the test group. The results of peripheal blood counts will be presented together with results from other experiments in order to show the chronology of changes up to 20 days after administration of cyclophosphamide.

RESULTS

The total and differential white blood cell count in normal animals and those given single intraperitoneal doses (300 mg/kg and 75 mg/kg) of cyclophosphamide are shown in Table I. After the 150 mg/kg dose (not shown in Table I), counts on groups of animals were performed only at 6 days (n=9, total white blood count 1 723 cells/µl, 95% mononuclear) and 9 days (n=8, total white blood count 3 375 cells/µl, 82% mononuclear, 0.4% basophil, 0.2% eosinophil and 17% neutrophil) after administration.

The leukopenia caused by all cyclophosphamide doses was most pronounced for the highest dosage, and most pronounced 3 to 6 days after administration. Total white cell counts had returned to near normal by day 9–10 after administration. At the highest dose, a slight leucocytosis in the recovery phase (after day 10) was seen, and was pronounced in occasional animals. At all doses a relative lymphocytosis occurred during the leukopenia

since granulocytes were markedly reduced in number. The effect on granulocytes was most marked 6 days after administration at all doses. A relative neutrophilia was seen in the recovery phase.

In Fig. 1, the changes in the "macroscopic score" (erythema and oedema) and the cell responses (test count minus normal) for the three doses of cyclophosphamide are shown and compared to control reactions. At the 300 mg/kg dose, both the macroscopic score and

Table I. *Total and differential white blood cell count in the peripheral blood of guinea pig*

Normal animals

	Total white blood cell count (WBC) cells/μl			Differential (%)							
				Mononuclear (Mono)		Basophil (Bas)		Eosinophil (Eos)		Neutrophil (Neut)	
N	Mean	SD	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range
23	4 600	1 367	2 200–8 200	62	41–81	0.5	0–2.5	4.6	1–11.5	32	14–54

Cyclophosphamide treated animals

Days after administration	300 mg/kg							
	WBC				Differential			
	N	Mean	SD	Range	Mono	Bas	Eos	Neut
1	5	3 480	923	2 300–4 700	36	0.2	1.2	63
3	9	1 166	475	340–1 700	72			
6	17	844	316	400–1 720	90			
9–10	5	4 128	1 777	2 320–7 000	76			
12–13	5	9 580	4 830	5 700–17 600	52	0	1.2	47
16–17	7	6 100	2 454	3 900–10 480	39	0	0.8	59
18	5	5 920	1 127	4 000–7 000	50	0.8	1.4	47
20	5	6 120	1 758	3 400–8 100	46	1.1	1.6	51

Days after administration	75 mg/kg							
	WBC				Differential			
	N	Mean	SD	Range	Mono	Bas	Eos	Neut
1	5	3 580	785	2 700–4 600	51	0.6	1.2	46
3	5	2 540	482	2 000–3 300	47	0	2.2	50
6	8	2 962	763	2 300–4 300	93	0	1.3	6
9–10	6	3 633	1 017	2 300–5 200	89	0	0.3	10
12–13	6	4 933	1 015	3 800–6 400	64	0	1.6	34
16–17	6	5 383	503	4 800–6 200	40	1.3	3.4	54
18	5	6 220	1 551	4 800–8 300	59	0.6	3.2	37
20	5	5 920	1 734	3 400–8 000	53	0.2	3.6	43

Cyclophosphamide was administered in a single dose intraperitoneally. When the WBC was low only polymorphonuclear/mononuclear differentiation was performed.

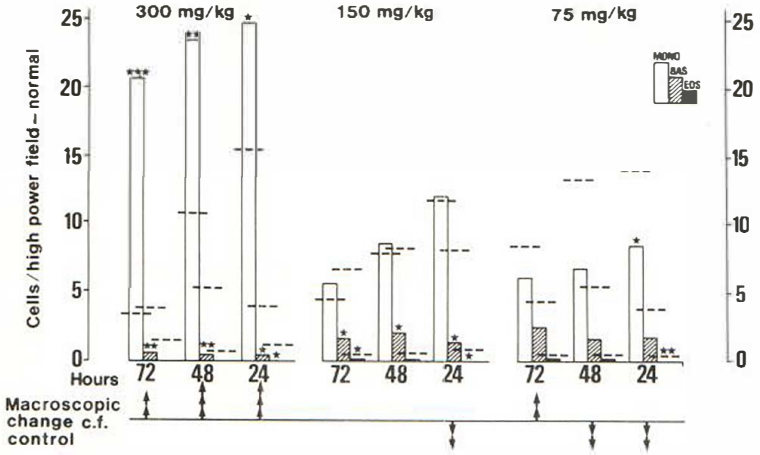

Fig. 1. Effect upon the allergic contact reaction to oxazolone of cyclophosphamide given in three single intraperitoneal doses (300, 150 or 75 mg/kg) to groups of five guinea pigs, six days prior to first test day. The dermal inflammatory cell response (test count minus normal) at 72, 48 and 24 hours (the chronological order in which the tests are applied-see Methods) for mononuclear (MONO), basophil (BAS) and eosinophil (EOS) cells in cells per high power field (average of 20 fields) are shown. Values for the control reaction are shown (---). Statistically significant changes compared to control are indicated by (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Levels of change in the macroscopic score compared to control reactions have been arbitrarily set (see Methods for criteria: ↑ = slight, ↟ = clear, ⇞ = marked).

the mononuclear cell response increased, while basophil and to a lesser degree eosinophil cell responses decreased.

At half the original dose (150 mg/kg), there was still a statistically significant reduction in the basophil and eosinophil cell response, though less marked than at the higher dose. The 24-hour reaction decreased in erythema and oedema. The mononuclear cell response was unchanged compared to the control reaction.

When the dose of cyclophosphamide was even smaller (75 mg/kg), the macroscopic score was decreased at the 24 and 48-hour reaction though not at the 72-hour reaction. All dermal cell counts were decreased but only statistically significantly so for the 24-hour mononuclear and eosinophil cell responses.

DISCUSSION

Previous reports have shown that cyclophosphamide causes a leukopenia after both high single (7) and continual doses (8, 9). The findings here presented confirm that a leukopenia occurs and show that it is dose-dependant. At the highest dose (300 mg/kg) the leukopenia is most pronounced 6 days after administration, somewhat later than previously reported (10). The differential white blood cell counts provide further informtion. All granulocyte numbers decreased, the effect being dose-dependant and most marked 6 days after administration. The neutrophil count showed a market "rebound" increase in a recovery phase which occurred after 10 days after administration. Even basophils showed a tendency to rebound. Within the time period of this experiment, eosinophils have barely returned to normal values—a rebound at a later point cannot be excluded.

The range of individual blood values is relatively large, a problem encountered in all peripheral blood counting. The phenomenon reflects individual variation between animals and probably even variations in the same animal from one time to another.

The effects of cyclophosphamide on macroscopic appearance and the dermal inflammatory infiltrate of the allergic contact reaction to oxazolone varied with dose. The highest dose increased the macroscopic score, the lowest dose decreased it. The mononuclear dermal infiltrate was increased at the highest dose, decreased at the lowest. Basophil and eosinophil dermal infiltrates decreased generally, the effect being least marked at the lowest dose. The relative absence of these granulocytes in the dermal infiltrate may reflect the low peripheral blood basophil and eosinophil counts. With the cyclophosphamide doses studied here, the macroscopic score would appear to follow the dermal mononuclear cell response, although we have previously seen that this coupling does not hold in all situations (6). At the point at which the present allergic contact reactions were induced (6 days after the administration), the 300 mg/kg dose had induced the most marked leukopenia with a relative monocytosis (lymphocytosis). Parodoxically, the mononuclear dermal cellular infiltrate of reactions was markedly increased at this dosage. At the 75 mg/kg dose, the fall in the peripheral white blood count at the same point was not as pronounced. The test reaction at this dose showed a decrease in the mononuclear dermal cell infiltrate. Thus recruitment of mononuclear cells to the allergic contact reaction would seem to be independent of quantitative peripheral white blood cell levels.

We have previously shown that cyclophosphamide has non-specific anti-inflammatory effects on the toxic contact reaction to croton oil (11). In the present experiment, the 75 mg/kg dose had an anti-inflammatory effect on the contact allergic reaction but it seems likely that this was non-specific rather than immunosuppressant in character. The higher dose of cyclophosphamide caused increased erythema and oedema and an increased mononuclear dermal cell infiltrate and must thus be the result of a nett augmentation of the allergic contact effector mechanism. It has been postulated that this may occur because T-suppressor lymphocytes with a comparatively rapid rate of turn-over, are particularly sensitive to cyclophosphamide (12). If this is so, then although the "immunosuppressant" cyclophosphamide can be said to have an "immunospecific" effect at higher doses, the nett result is augmentation of the allergic contact reaction.

In any discussion of the effects of cyclophosphamide (and other immnosuppressants) on contact allergy and cell-mediated immunity in general there is still a great need for background information. Our guinea pig model has provided quantitative and qualitative information about the inflammatory cell infiltrate in the dermis of allergic contact reactions. Extrapolation of results from experimental animal models to the clinical situation in man must always be done with due regard to species specificity. The peripheral blood findings presented in this paper make possible some dose comparisons between guinea pig and man. To judge from the peripheral blood effects, the extremely high 300 mg/kg cyclophosphamide dose which has enjoyed almost traditional use in animal experimentation must, also in man, approximate the very highest doses in clinical use. The 75 mg/kg dose in guinea pig would appear to correlate to a more relevant single dose in man. At this lower dose, clear anti-inflammatory effects have been exerted on the allergic contact reaction. We have, however, no direct evidence that this effect is other than non-specific in nature.

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