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INFLUENCE OF ^{90}Sr, ADULT THYMECTOMY AND ANTILYMPHOCYTEGLOBULINE ON T-CELLS IN MOUSE PERIPHERAL BLOOD

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Abstract

Three groups of male CBA mice were treated by (1) ^{90}Sr injection (14.8 kBq/g body weight i.p.), (2) adult thymectomy (ATx) + antilymphocyteglobuline (ALG), and (3) ^{90}Sr + ATx + ALG, respectively, and one untreated group served as a control. The relative number of T-lymphocytes was determined in peripheral blood mononuclear cells of individual animals using a dye exclusion cytotoxicity assay with antisera against the T-cell surface membrane marker Thy 1.2 and complement. Total and differential leucocyte counts were also performed. ^{90}Sr irradiation decreased the total number of leucocytes irrespective of type, and the combined treatment of ATx and ALG decreased mainly mononuclear cells and particularly T-cells. The most advanced T-cell depletion in peripheral blood was found in the ^{90}Sr + ATx + ALG treated group with a 97 per cent reduction as compared with untreated controls. ATx + ALG thus proved to be useful for blood T-cell depletion in mice treated simultaneously with ^{90}Sr, and might provide a valuable tool in investigations on the possible role of cell-mediated immune response in radiation-induced oncogenesis, with particular emphasis on selective depletion within the monomorphonuclear compartment.

The cell-mediated immune system has gained much attention among the factors involved in radiation-induced tumour development. This investigation of T-cell distribution in the peripheral blood of adult thymectomized (ATx) mice exposed to ^{90}Sr and treated with antilymphocyteglobuline (ALG) constitutes an integrated part of a project concerning the role of immunologic responsiveness in radiation-induced malignancies. The basic experiments of this project, presently under way, were designed to analyse the influence of immunosuppression by ATx or ALG, or both, in mice treated with ^{90}Sr, as well as by ^{90}Sr itself, on the development of tumours. This is of interest since (a) ionizing radiation is known to decrease the immunologic reactivity (11), (b) ^{90}Sr-induced osteosarcomas have been reported to possess specific transplantation antigens (4, 6), and (c) unspecifically increased immunologic responsiveness (Bacillus Calmette-Guérin; BCG) decreases the incidence of these tumours (7). Evaluation of the above experiments required information on the functional capacity of the immune system, particularly the cell-mediated part. Thus a T-cell enumeration in peripheral blood was considered to be of value and this paper gives the results of a cytotoxicity assay for T-cells performed on separated peripheral blood mononuclear cells (PBMNC). In subsequent reports, in vitro lymphocyte responsiveness to mitogens, skin-allograft rejection, and phagocytic function of the reticuloendothelial system (RES) in similarly treated mice, will be described.

Materials and Methods

Animals. Specific pathogen free (SPF) inbred CBA male mice were obtained from the Anticimex breeding laboratories (origin: CBA/Ca via Harwell

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Table 1

Total and differential leucocyte counts and calculated number of T-cells per μl peripheral blood, mean ± SE. Per cent T-cell reduction and non-T-cell reduction, versus untreated controls. (p) = Student's t-test p-value for test group versus control group*

	Group code			
	D I	D II	D IV	D VII
Treatments	Untreated control	^{90}Sr	^{90}Sr + ATx + ALG	ATx + ALG
No. of mice	11	10	9	11
Total No. of leucocytes	6 236±642	1 810±198	1 072±189	3 800±663
Polymorphonuclear cells	36%	36%	64%	41%
	2 227±315	650±122	683±155	1 555±499
		(0.001>p)	(0.001>p)	(0.30>p>0.02)
Monomorphonuclear cells	64%	64%	36%	59%
	4 009±584	1 160±107	389±59	2 245±255)
		(0.001>p)	(0.001>p)	(0.02>p>0.01)
*Calculated**				
No. of T-cells	2 045	731	70	382
Per cent reduction of T-cells versus D I		64	97	81
Per cent reduction of non-T-cells versus D I		78	84	5

* Calculation based on mean net percentage of T-cells in PBMNC recorded in Table 2.

and Stockholm University) (Evelund, 191 71 Sollentuna, Sweden) and during the time of the experiment were housed in plastic film isolators (Metall & Plastic, 7760 Radolfzell, West Germany) under sterile technical conditions. The animals were maintained in macrolon II cages, 5 in each, and given free access to a sterilized pelleted diet (R3-feed for rats and mice, Astra-Ewos, 151 27 Södertälje, Sweden) and water. Air temperature was 22° to 24°C and relative humidity 50 to 55 per cent. The light–dark cycle was 12/12 h.

Surgery. Thymectomy was performed under pentobarbital (Mebumal vet.) anaesthesia by a median incision over the anterior thorax and neck (10). The anterior thoracic orifice was widened by a short median slit in the sternum. The thymic lobes were removed by suction, and the wound immediately closed by stainless steel clips (Clay Adams). The successful removal was first checked by examination of the collected lobes under magnification and rechecked at autopsy. The non-thymectomized mice were sham-treated.

Nuclide. Carrier-free strontium-90 nitrate in l-N nitric acid, supplied by Amersham International, Amersham, U.K., was diluted in saline to an activity of 1.69×10^3 kBq (45.75 μCi) ^{90}Sr per ml, and in equilibrium with ^{90}Y when injected.

ALG. Freeze-dried horse-antimouse-lymphocyteglobuline, obtained from TNO, Rijswijk Z.H., The Netherlands, was dissolved in saline to a concentration of 30 mg/ml.

Antisera. Anti-Thy 1.2 serum was produced by immunizing AKR/J mice eight times with CBA/J thymocytes (8). Rabbit serum (1 : 8) preabsorbed to normal mouse spleen cells served as the source of complement.

Statistics. Student's t-test was used to analyse the results recorded in Table 1.

Experimental procedures. The animals were randomly divided into four groups, coded in accordance with parallel investigations and subjected to ATx, internal exposure to ^{90}Sr and long-term treatment with ALG in combinations as follows: Group D I, untreated control; group D II, ^{90}Sr; group D IV, ^{90}Sr + ATx + ALG; group D VII, ATx + ALG.

Adult thymectomy was performed at 60±3 days of age. ^{90}Sr was administered by the route of intraperitoneal injection at the age of 75±3 days and weight 25±2 g. The amount injected was 14.8 kBq (0.4 μCi)/g body weight.

Table 2

Net percentage of T-cells in PBMNC of CBA mice, determined by cytotoxic assay. Each value represents one individual

	Group code				No. of weeks ALG injected
	D I	D II	D IV	D VII	
Exp. No.					
1	67	76	7	8.5	11
2	49	60	*	6	11
3	48	**	21	31	15
4	41	57	13	11	15
5	51	64	20	27	19
6	50	59	29	15	19
7	54	*	**	23	19
Mean ± SE	51±3.0	63±3.4	18±3.7	17±3.7	

* Animal lost owing to radiation induced malignant lymphoma.
** Value missing owing to technical failure in blood sampling.

Antilymphocyteglobuline was administered intraperitoneally in doses of 6 mg/mouse/week, beginning 172 days after exposure to ^{90}Sr.

Blood samples for T-cell enumeration were taken after 11, 15 or 19 weeks of ALG treatment (Table 2), and in each case carried out two days after the last injection of ALG. Leucocyte counts were similarly performed in equally treated mice.

Processing of blood. Blood samples for leucocyte counting were obtained by puncture of the orbital venus plexus after ether anaesthesia. Mono- and polymorphonuclear cells were stained with Türk's gentian violet and counted in a Bürker haemocytometer.

In order to prepare individual peripheral blood mononuclear cell (PBMNC) suspensions from each mouse, one eye was excised and 1 to 2 ml of blood collected in a vial-tube containing 2 drops of heparin (Vitrum, 5000 IU/ml). An equal volume of phosphate buffered saline (PBS) was added. On each occasion blood samples from four animals representing the four differently treated groups were handled simultaneously.

The mononuclear cells were separated from the red blood cells and polymorphonuclear leucocytes on Ficoll-Hypaque gradients (2), washed twice in phosphate buffered saline, resuspended in Hank's balanced salt solution (HBSS) and brought to an equal concentration of 1 to 2×10^6 cells/ml. Viability was determined by trypan blue exclusion staining, and throughout the experiments exceeded 95 per cent.

Enumeration of T-lymphocytes. The identification and enumeration of T-lymphocytes was carried out by the cytotoxicity technique using antisera against the characteristic T-cell surface membrane marker Thy 1.2 in the presence of complement. PBMNC suspensions plus antiserum, in like volumes of 50 or 100 µl, were incubated in a V-shaped microtitration plate (Titertec) for 45 min at +4°C. Hank's balanced salt solution was added in surplus. The cells were spun down and after removal of the supernatant vibrated in the residual liquid.

Complement, diluted to an optimal concentration with Hank's balanced salt solution, was added in a volume of 50 µl and incubation continued at +37°C for 30 min until stopped by cooling on ice. Control cultures without antiserum, containing only complement, were run in parallel. The cultured cells were stained with trypan blue and the ratio of non-vital and vital peripheral blood mononuclear cells was determined by counting a minimum number of 100 cells. Owing to inevitable death of cells from other reasons than susceptibility to anti-Thy 1.2 serum, the relative proportion of T-cells in test culture was calculated as net percentage of T-cells (per cent non-vital cells in test culture minus per cent non-vital cells in control culture). The average percentage of dead cells in control cultures was 13±2 (SE).

Results

The data obtained (Table 1) indicate that irradiation with ^{90}Sr decreased the number of monomorphonuclear and polymorphonuclear cells in equal proportions, and significantly as compared with untreated controls, which is in good agreement with the experience of others (3, 5). ATx + ALG treatment decreased the monomorphonuclear cells almost significantly, but not the polymorphonuclear cells. The strongest deficit of monomorphonuclear cells was found in group D IV treated by ^{90}Sr + ATx + ALG, which was significant as compared with each of the other groups (D II versus D IV, $0.001>p$).

The relative proportion of T-cells in PBMNC suspensions (Table 2) was about 18 per cent in the ATx + ALG group as well as in the ^{90}Sr + ATx + ALG group, which was strikingly lower than was found in the control group (51%) and the ^{90}Sr group (63%).

The net number of T-cells in peripheral blood,

calculated on the net percentage of T-cells in PBMNC and the total number of monomorphonuclear cells per μl peripheral blood, is shown in Table 1. The reduction of T-cells was obvious in all treated groups, and particularly striking in the ^{90}Sr + Tx + ALG group, with 97 per cent reduction as compared with controls. The ^{90}Sr treated group and the ATx + ALG treated group reached a 64 and 81 per cent reduction, respectively. The non-T mononuclear cell population was strikingly reduced by ^{90}Sr (78%), in contrast to the results with ATx + ALG.

Discussion

Thy-antigen is documented to be a useful and reliable marker for mouse T-cells (8, 9). For easiest possible enumeration of blood T lymphocytes in individual mice the anti-Thy 1.2 cytotoxicity test was used to determine the ratio of T and non-T cells in separated PBMNC. The number of T-cells in peripheral blood could thus be calculated after total and differential leucocyte counts.

This method admittedly only gives the approximate number of T-cells but it is nevertheless considered useful for comparative evaluation of the influence of ^{90}Sr and ATx + ALG on the T-cell population.

Internal exposure to ^{90}Sr, as well as ATx + long term ALG treatment, showed a strong depleting effect on the PBMNC fraction, and the combined treated (D IV) enhanced the depletion significantly. ^{90}Sr reduced the T-cells by 64 per cent as compared with controls. ATx + ALG induced an 81 per cent reduction, and the combined treatment showed the most pronounced reduction of T-cells, by 97 per cent, i.e. 70 cells left per μl blood.

It must be noted, however, that the different treatments due to different modes of action as consequences on cell regeneration do not necessarily result in depletion of congruent T-cell fractions.

The extreme T-cell depletion in group D IV is attributed to the continuous exposure of the bone marrow to ^{90}Sr irradiation in combination with the lack of cells for recruitment following ATx + ALG.

ATx + ALG treatment alone (D VII) reduced non-T-cells only to a minor extent (5%), if at all, and this stands in great contrast to ^{90}Sr treatment which reduced the non-T-cells preferentially (78%). The pronounced depletion of T-cells in the ATx + ALG treated groups described corresponds well with preliminary results on impaired in vitro responsiveness of spleen cells to mitogens (PHA and Con A) as well as inability to reject skin allografts after the same pretreatments (1).

The conclusion reached is that ATx + ALG treatment might provide a valuable tool in investigations on the role of T-cell or T-cell dependent responsiveness in radiostrontium-induced oncogenesis, particularly since this treatment in our experience does not affect the survival time and consequently facilitates comparative analysis of the tumour development.

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