QUANTITATIVE STUDIES ON THE BEHAVIOR OF SENSITIZED LYMPHOCYTES IN VITRO

II. Inhibitory Influence of the Immune Suppressor, Imuran, on the Destructive Reaction of Sensitized Lymphoid Cells Against Homologous Target Cells*

By DARCY B. WILSON,‡ Ph.D.

(From the Wistar Institute of Anatomy and Biology, and the Department of Medical Genetics, University of Pennsylvania, Philadelphia)

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On the basis of results presented in Part 1 of this series (1) it was suggested that the destructive effect of sensitized lymphocytes upon homologous cells in tissue culture depends, in part, on an active metabolism of the lymphoid cells. To provide further information on this point, the experiments to be described were conducted to ascertain whether the antimetabolite imuran, an imidazole derivative of 6-mercaptopurine, has any influence on the cytocidal and cytostatic activities of sensitized lymphoid cells against homologous target cells *in vitro*.

Materials and Methods

The procedures and system used to assay the destructive effect of specifically sensitized lymphoid cells on homologous target cells have been described before (1). To determine the effect of imuran on this destructive reaction, known numbers of lymphoid cells were suspended in growth medium with or without the drug and then added to Leighton tube cultures of Lewis target tumor cells of the Le-1 line. The number of these target cells remaining attached to glass at the end of a 48 hour culture period was determined with an electronic particle counter or by hemocytometer counts of cell nuclei.

A stock solution of imuran[1] was prepared by dissolving 100 mg of the drug in 20 ml of sterile distilled water and raising the pH to 10 with 1 N NaOH; this solution was kept at 4°C pending use.

Treatment of the Data.—To facilitate comparisons of the data of the various experimental groups, the results are expressed as "the percentage of target cell survival"; *i.e.*, the number of cells surviving in cultures with sensitized lymphoid cells × 100, divided by the number surviving in cultures with normal lymphoid cells.

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‡ Fellow of the Helen Hay Whitney Foundation. Present address: Department of Medical Genetics, University of Pennsylvania, Philadelphia.

[1] Imuran, brand azathioprine, Lot AN 44763. The author wishes to thank Dr. Geo. Hitchings of Burroughs Wellcome and Co., Tuckahoe, New York for supplying this drug.

RESULTS

It was essential for these studies to determine the concentration range of imuran which was without demonstrable toxic influence on lymphoid cells under the culture conditions employed. To do this, known numbers (2 to 5 million) of normal or sensitized lymph node cells were incubated in growth medium con-

TABLE I

Effect of Varying Concentrations of Imuran on the Viability of Lewis Target Cells and Lymphoid Cells Derived from Normal or Immunized DA Rats

Cells	Mean* survival of cells after 48 Hrs in media containing imuran (μg/ml)					
	100	10	5	1	0.1	None
Normal DA L.N. cells (per cent)	21.0‡	23.8		59.1	59.3	56.8
		55.4		68.4		87.8
			62.6	63.3		59.6
			53.6			58.5
				57.9		65.9
	13.4	28.1	56.2	56.6	59.9	60.1
	3.9	4.8	14.2	26.0	33.8	27.8
Sensitized DA L.N. cells (per cent)	9.8	21.7		34.7	39.9	38.5
		33.2		70.6		77.6
			18.6	12.1		15.0
			56.7			61.5
	11.2	26.4	51.3	52.3	59.7	60.3
		34.8		81.8		88.2
Lewis target cells ($\times 10^3$)	16	96		219	349	347
	30	84	160	270	401	423
	4	84	151	410	473	469

L.N., lymph node.

* Means in this table based on counts of 3 to 4 tubes in each group.

‡ Per cent lymphocyte survival, $\frac{\text{No. cells excluding trypan blue at end of culture} \times 100}{\text{No. viable cells placed into culture initially}}$

taining varying concentrations of the drug. At the end of 48 hours, the number of viable lymphoid cells, those which excluded trypan blue dye at a final concentration of 0.05 per cent, was determined by phase contrast microscopy. The results, presented in Table I, show that concentrations of imuran up to 5 μg/ml did not influence the viability of normal or immune lymphoid cells over the exposure period employed. For some as yet unexplained reason, the number of sensitized lymphocytes which survived the incubation period tended to be variable. However, the inconsistency was not due to imuran since it was also observed in tubes which did not contain the drug.

The effect of the drug on target cells was determined by adding fresh medium containing specified concentrations of imuran to 20-hour Leighton tube cultures of Le-1 target cells. These cultures were reincubated for 48 hours at which time the number of cells present was determined. The results, based on the number of target cells surviving, indicated that imuran was non-toxic to growing populations of these cells at low concentrations (0.1 μg/ml). Up to a concentration of 10 μg/ml this apparent toxic effect was not one of destruction, but rather of growth inhibition; levels of 5 to 10 μg/ml were, therefore, considered tolerable for the target cells.

TABLE II

Inhibitory Influence of Imuran on the Destruction of Homologous Target Cells by Sensitized Lymphoid Cells

Group	Concentration imuran	Mean No. target cells surviving after culture			Per cent* target cells remaining
		No L.N. cells ($\times 10^3$)	Normal L.N. cells ($\times 10^3$)	Sensitized L.N. cells ($\times 10^3$)	
	μg/ml				
A (non-irrad. target cells)	0	151.6	508.0	39.6	7.8
	1	101.8	237.0	81.6	36.3
	5	73.8	101.4	71.8	70.9
B (irrad. target cells)	0	276.0	233.2	149.2	64.0
	1	300.0	242.8	192.0	79.1
	5	284.0	208.1	196.0	94.2
	0	101.1	97.9	56.7	57.9
	1	101.7	105.5	70.0	66.3
	5	99.8	101.1	83.3	82.4

* $\frac{\text{No. target cells remaining after exposure to sensitized L. N. cells} \times 100}{\text{No. target cells remaining after exposure to normal L.N. cells}}$

The influence of imuran on the cytocidal capacity of sensitized lymphoid cells was determined by including this drug in the medium (1 to 5 μg/ml) in which target cells and attacking lymphocytes (5 million) were cultured. The results (Table II, group A) showed that, in the presence of imuran a smaller number of the target cells was destroyed by the activities of the sensitized lymphoid cells. The mean per cent survival of target cells in this experiment was proportional to the concentration of imuran employed.

Since the drug had a demonstrable inhibitory effect on the growth of the target cells, this experiment was repeated using target cells rendered incapable of division by x-irradiation with 2000 roentgens. The results were similar (Table II, group B), in that imuran, at concentrations commensurate with the viability of lymphocytes, greatly reduced the destructive effect of sensitized lymphocytes on homologous target cells *in vitro*.

An experiment was then performed to determine whether the *continuous* presence of imuran was necessary to suppress the destructive capacity of sensitized lymphoid cells. Normal or sensitized lymphoid cells were suspended in media alone or in media supplemented with 1 or 10 μg imuran/ml. These cells were incubated in centrifuge tubes for 1 hour at 37°C, at the end of which time they were spun down. The cells of group A, incubated in media, were resuspended in fresh media, while those of groups B and C, which had been incubated in media containing imuran, were resuspended in media with (B) or

TABLE III

In Vitro Reactivity of 5 × 10^6 Sensitized DA Lymph Node Cells Treated with Imuran before and during Incubation with Lewis Target Cells

Group	Conditions		Mean No. and per cent difference target cells surviving			
	Imuran	Lymph node cells	Experiment 1		Experiment 2	
			(× 10^3)	per cent	(× 10^3)	per cent
	$\mu g/ml$					
A	0	Normal	648.3	45.9	285.0	53.8
		Sensitized	297.6		153.4	
B (during culture)	1	Normal	335.6	64.1	145.2	69.0
		Sensitized	215.1		100.2	
	10	Normal	168.1	97.7	116.3	94.6
		Sensitized	164.2		110.2	
C (before culture)	1	Normal	496.3	54.1	240.3	58.3
		Sensitized	268.3		140.1	
	10	Normal	500.4	66.8	238.3	54.6
		Sensitized	280.6		130.1	

without the drug (C). The results of two such experiments, (Table III) with non-irradiated Lewis target cells, showed that inhibition of the destructive capacity of sensitized lymphoid cells was dependent upon the continued presence of the drug; *i.e.*, preexposure of lymphoid cells for 1 hour with the drug failed to inhibit the immunological activity of lymphoid cells over a subsequent 48 hour culture period with the homologous target cells.

DISCUSSION

Purine analogues have been widely used both experimentally and clinically because of their striking effect on the course of the immune response (See references 2, 3); presumably, these drugs act through an interference with RNA

metabolism. Administration of 6-mercaptopurine concomitantly with bovine serum albumin in rabbits has resulted in complete non-reactivity to the antigen. However, the drug had little or no effect when it was given at the height of the immune response (4). For this reason, it is believed that inhibition accompanying the use of 6-mercaptopurine and other purine analogues is concerned principally with the initial metabolic processes leading to the immune response rather than with the production or release of antibody itself. The results of the present studies have shown that the continuous presence of one of these RNA inhibitor drugs in small quantities serves to drastically reduce the cytocidal activity of specifically sensitized lymphoid cells *in vitro*. This inhibitory effect on the immunological activities of a population of lymphocytes containing already sensitized cells suggests that an RNA-dependent process essential for the cyto-destructive reactivities of these cells was interrupted. Because imuran is thought to affect RNA-mediated protein synthesis, and not pre-formed proteins, these results make it unlikely that the destructive activities of sensitized lymphoid cells, at least *in vitro*, depend strictly on pre-formed antibodies located on or within lymphoid cells. Furthermore, that an interaction between target cells and activated node cells might involve active protein synthesis is also suggested by evidence that the transfer reaction in rabbits can be temporarily abolished following treatment of sensitized lymphoid cells with RNase (5).

Using hydrocortisone in their culture media, Rosenau and Moon (6) showed that while the lytic activities of specifically sensitized splenic cells upon homologous target L cells could be partially inhibited, the drug did not prevent aggregation of the lymphocytes around the target cells. It is conceivable that this steroid-induced inhibition of the destructive activities of sensitized lymphocytes may result from an interference with protein metabolism in the attacking lymphocytes. However, cortisone and related steroids have also been known to stabilize lysosomal membranes against induced permeability changes (7), a factor which might contribute to the inhibition of the immunological activities of sensitized lymphocytes.

SUMMARY

The cytocidal effect of specifically sensitized lymphoid cells on homologous target cells in culture can be inhibited by small quantities of an imidazole derivative of 6-mercaptopurine (imuran). This inhibition takes place only in the continuous presence of this drug, and at concentrations which apparently do not affect the viability of the attacking lymphoid cells. These results seem to support the contention that RNA-dependent protein synthesis on the part of the lymphoid cells is necessary for a destructive interaction between sensitized node cells and homologous target cells *in vitro*.

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