SUPPRESSION OF DELAYED HYPERSENSITIVITY IN VITRO BY INHIBITION OF PROTEIN SYNTHESIS*

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Previous studies have demonstrated that peritoneal exudate cells obtained from guinea pigs exhibiting delayed hypersensitivity are inhibited from migrating out of capillary tubes by the specific sensitizing antigen. This reaction appears to be an intimate property of the cells and is not associated with circulating antibody (1, 2). In experiments with mixed populations of cells it was found that only a few sensitive cells are required to cause a normal population of cells to be inhibited by antigen. This effect of sensitive cells on normal cells was abolished if the sensitive cells were heated or disrupted by freezing and thawing (3). Attempts to sensitize normal cells with extracts of sensitive cells or sera taken from sensitive animals have failed. These observations suggested that the specific inhibition of cell migration by antigen was dependent on active cellular metabolism.

The present article will be concerned with the effect of selected inhibitors of protein and nucleic acid synthesis on the delayed hypersensitivity reaction *in vitro*.

Materials and Methods

Sensitization.—Delayed hypersensitivity was induced in Hartley strain guinea pigs by the injection of antigens incorporated in complete Freund's adjuvant (2).

Antigens.—Purified protein derivative (PPD) Merck, Sharpe, and Dohme, West Point, Pennsylvania. Dinitrophenyl-bovine gamma globulin (DNP-BGG) was prepared by the method of Benacerraf and Levine (4).

Inhibitors.—Puromycin and 3′ aminonucleoside of puromycin (Nutritional Biochemical Corporation, Cleveland) were dissolved in water, and sterilized by filtration through Millipore filters. They were incorporated into tissue culture media in the following range of concentrations: puromycin, 0.5 μg to 10 μg/ml, 3′ aminonucleoside, 1 μg to 100 μg/ml. Three analogues of puromycin (kindly supplied by Dr. Leon Goldman of Lederle Laboratories), L-phenylalanyl, L-tyrosyl, and L-tryptophanyl, were solubilized by the addition of two equivalents of HCL and treated as above; final concentrations ranged from 5 μg to 100 μg/ml.

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Actinomycin D (kindly supplied by Dr. Rody Cox) and chloramphenicol were used in concentrations as follows: actinomycin, 0.1 to 10 μg/ml; chloramphenicol, 1.0 μg to 10 mg/ml.

In Vitro Test for Delayed Hypersensitivity.—The methods used for placing the cells in culture, based on those of George and Vaughan (1), have been described in detail elsewhere (1, 2). In brief, peritoneal exudate cells, induced by oil, are washed, made up in tissue culture medium, centrifuged in capillary tubes, and the portion of the tubes containing the packed cells cut and placed in Mackaness type chambers (two tubes per chamber). In each experiment chambers were filled with media as follows: (*a*) two chambers for each dilution of inhibitor to be tested; (*b*) two chambers for each dilution of inhibitor plus antigen; and (*c*) four chambers for control media, two with and two without antigen. Unless otherwise stated the concentrations of antigens were as follows: PPD, 15 μg/ml; DNP-BGG, 100 μg/ml. The chambers were incubated for 24 hours at 37°C. During this period the cells migrated out of the tubes onto the glass. The area of migration was projected, drawn, and measured with a planimeter. Data was calculated using the following formula:

$$\frac{\text{Area of migration with antigen}}{\text{Area of migration with no antigen}} \times 100 = \text{per cent migration with antigen}$$

Incorporation of C^{14}-Leucine or Uridine into Peritoneal Cells.—Flasks were prepared as follows: 50 ml of minimal essential medium (Eagle) containing 15 per cent guinea pig serum and 1×10^6 peritoneal cells/ml in each. In studies with puromycin or chloramphenicol C^{14}-leucine, 0.5 μc, was added to each flask. Puromycin was used in concentrations of 10 μg/ml and 0.5 μg/ml; the concentrations of chloramphenicol were 500 μg/ml and 50 μg/ml. In actinomycin studies 0.5 μc of C^{14}-uridine was added to each flask and the concentrations tested were 10 μg/ml and 0.1 μg/ml. In all groups control flasks were identically prepared except that no antimetabolites were added. The flasks were incubated in a shaker at 37°C. Samples were removed at intervals, and the cells centrifuged and washed twice in saline. The cells were lysed in 0.5 per cent sodium deoxycholate (5). The lysate was precipitated with 10 per cent trichloracetic acid and the spun precipitate dissolved in 1 N sodium hydroxide. An aliquot was added to Brays scintillation counting solution (6) and radioactivity counted in a Packard tricarb scintillation counter. Protein determinations (7) were carried out and specific activities determined. The above procedures were carried out with Dr. Rody Cox and the methods used are described in detail by Griffin and Cox (8).

RESULTS

Effect of Puromycin on Sensitive Cells.—The migration *in vitro* of peritoneal exudate cells taken from guinea pigs exhibiting delayed hypersensitivity is consistently inhibited by the specific sensitizing antigen. The results of experiments in which puromycin was present during the migration of such cells are presented in Table I and Fig. 1. When puromycin was present in the media at a concentration of 10 μg/ml the cells were no longer inhibited by antigen. In three experiments the average migration in these chambers was 88 per cent, while control cells (without puromycin) migrated only 17 per cent in antigen. The average migration of cells exposed to 5 μg/ml of puromycin in the presence of antigen was 60 per cent. When the concentration of puromycin was lowered to 0.5 μg/ml no effect of puromycin was seen and the cells were inhibited by antigen.

It was observed that 10 μg/ml of puromycin itself produced some inhibition

of migration. In order to illustrate this, the absolute areas of migration are presented in Table II. It can be seen that cells in 10 μg/ml of puromycin migrated only half as far as those in control media. Nevertheless, at this concentration of puromycin plus antigen the cells migrated two to six times further than in antigen alone. Thus puromycin, at 10 μg/ml, significantly decreases the effect of antigen on the migration of sensitive cells despite its concomitant effect on migration *per se*. However, in the presence of 5 μg/ml of puromycin, cells migrated as well in 24 hours as those in normal media; this concentration of puromycin is still sufficient to significantly suppress the inhibiting effect of antigen.

Effect of Puromycin on Uptake of C^{14}-Leucine.—Further studies were carried out to determine whether the concentration of puromycin that interfered with the *in vitro* reaction of sensitive cells and antigen could significantly inhibit

TABLE I

The Effect of Puromycin on the Migration of Tuberculin-Sensitive Cells in the Presence of PPD

Experiment	Migration in antigen*			
	No puromycin	10 μg	5 μg	0.5 μg
	per cent	*per cent*	*per cent*	*per cent*
1	5	69	40	16
2	26	96	68	25
3	20	100	71	10
Average.....	17	88	60	17

* PPD 15 μg/ml.

protein synthesis by peritoneal exudate cells, as measured by the incorporation of C^{14}-leucine into protein. After 3 hours of incubation in 10 μg/ml of puromycin there was an 81 per cent inhibition of C^{14}-leucine uptake by the cells into precipitable protein. 0.5 μg/ml had no effect (see Fig. 2). These results correlate well with the findings in cell migration studies where 10 μg/ml of puromycin reduced inhibition of migration by antigen and 0.5 μg/ml was ineffective. Thus, the concentration of puromycin which prevented the inhibition of cell migration was also sufficient to suppress protein synthesis by the cells.

Effects of Puromycin Analogues.—Additional evidence that the action of puromycin was clearly a result of its specific effect on protein synthesis was obtained with analogues of puromycin. In a series of experiments on the structural requirements for the activity of puromycin, Nathans and Neidle (9) have shown that the ability of puromycin to inhibit protein synthesis could be abolished by substituting certain amino acids for the L-o-methyltyrosine on the molecule. They showed that the L-phenylalanyl and L-tyrosyl analogues were

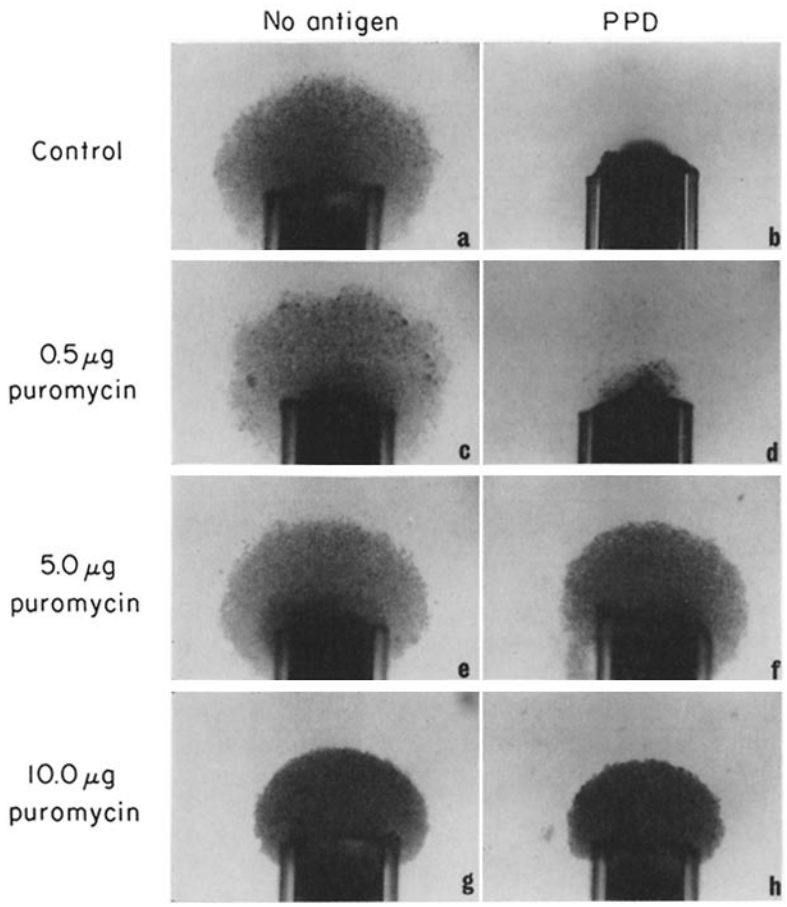

FIG. 1. Effect of puromycin on the inhibition of migration by antigen. All capillaries photographed are from the same pool of tuberculin sensitive cells. (*a*) Cells in normal media; (*b*) cells in presence of 15 μg/ml PPD; (*c*) cells migrating in the presence of 0.5 μg puromycin; (*d*) cells migrating in the presence of 0.5 μg puromycin and 15 μg/ml PPD; (*e*) cells migrating in 5.0 μg/ml puromycin; (*f*) cells in 5.0 μg/ml puromycin and 15 μg/ml PPD; (*g*) cells migrating in 10.0 μg/ml puromycin; and (*h*) cells in 10.0 μg/ml puromycin and 15 μg/ml PPD.

Photographs taken after 24 hours of incubation. × 35.

potent inhibitors of protein synthesis while L-tryptophanyl or the 3′ aminonucleoside of puromycin were inactive.

These four analogues were tested in the *in vitro* system of cell migration. The results of these experiments (shown in Table III) demonstrate that the analogues which inhibit protein synthesis (L-phenylalanyl and L-tyrosyl) also prevent the inhibition of migration by antigen. In contrast, the analogues (L-

tryptophanyl and 3′ aminonucleoside of puromycin) which do not inhibit protein synthesis had no demonstrable effect on the migration of sensitive cells.

Effects of Actinomycin D.—The results of the studies described above indicated that active protein synthesis was necessary for the inhibition of cell migration by antigen to occur. It was of interest to determine whether the same effects could be obtained with actinomycin D, an inhibitor which is known to interfere with DNA dependent RNA synthesis. The results of these experiments are shown in Table IV. Concentrations of 10 μg/ml of actinomycin in the culture media consistently decreased the effect of antigen on sensitive cells. 1 μg/ml of actinomycin was effective in half the experiments while 0.1 μg/ml

TABLE II

The Effect of Puromycin on the Migration of Tuberculin-Sensitive Cells in the Presence of PPD (Expressed as Absolute Area of Migration)*

Experiment	Migration, mm²							
	No puromycin		10 μg		5 μg		0.5 μg	
	Control	PPD	Control	PPD	Control	PPD	Control	PPD
1	5.8	0.3	2.9	2.0	4.7	1.9	4.9	0.8
2	4.3	1.1	2.4	2.3	4.7	3.2	4.8	1.2
3	3.9	0.8	2.1	2.1	4.5	3.2	4.8	0.5
Average.....	4.7	0.7	2.4	2.1	4.6	2.8	4.8	0.8

* PPD 15 μg/ml.

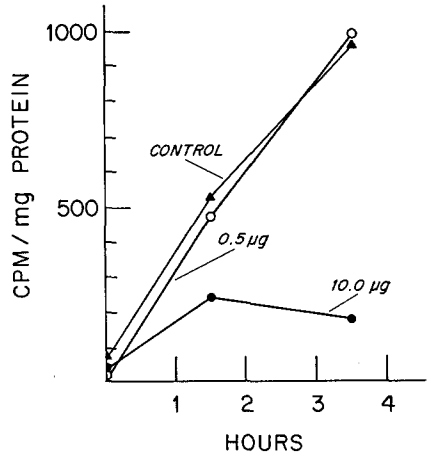

FIG. 2. Effect of puromycin (10 μg/ml and 0.5 μg/ml) on the incorporation of C^{14}-leucine into precipitable protein by peritoneal cells.

in two experiments produced no effect on the inhibition of migration by antigen. A set of chambers were prepared to contain the same minute concentrations of alcohol used in dissolving the actinomycin; no effect of alcohol was seen on cell migration or inhibition by antigen.

It was also observed in these experiments that actinomycin produced toxic effects during the first 18 hours. Cells in the presence of actinomycin often migrated less than those in control media when no antigen was present, and all migration had ceased by 18 hours. Though this toxicity was most often seen at 10 μg/ml it appeared erratically with all concentrations tested. Despite this background of toxic effects of actinomycin, the sensitive cells migrated two to

TABLE III

*The Effect of Analogues of Puromycin on the Migration of Tuberculin-Sensitive Cells in the Presence of Antigen**

Compound	Concentration	Migration with antigen
	M	*per cent*
None	—	10
Puromycin	2×10^{-5}	67
L-phenylalanyl	1×10^{-4}	85
	2×10^{-5}	67
L-tyrosyl	2×10^{-4}	74
	2×10^{-5}	11
L-tryptophanyl	2×10^{-4}	11
3'Aminonucleoside	2×10^{-4}	15

* 15 μg PPD/ml.

ten times further in actinomycin with antigen than when exposed to antigen alone.

Actinomycin D (in concentrations of 10 μg/ml) reduced C^{14}-uridine incorporation into acid precipitable material by the cells by 84 per cent following 3 hours' incubation. At a lower concentration (0.1 μg/ml) the uptake was still inhibited by 50 per cent. As was shown above, 10 μg/ml actinomycin consistently allowed sensitive cells to migrate further in antigen while no effect on the interaction of antigen and sensitive cells was seen with 0.1 μg/ml actinomycin. The partial reduction of RNA synthesis by actinomycin at 0.1 μg/ml may still allow protein synthesis to proceed at a relatively normal rate. It is of interest, in this regard, that enzyme induction can still occur normally despite a moderate reduction in RNA synthesis by actinomycin in other systems studied (8).

Effects of Chloramphenicol.—Chloramphenicol was also assayed for its effect

on the inhibition of cell migration by antigen. In four experiments chloramphenicol was added to the culture media of sensitive cells in concentrations ranging from 10 mg to 0.1 μg/ml. Chloramphenicol, 10 mg/ml, produced a toxic effect on the cells. At concentrations of 1000 μg/ml or less, the inhibition of migration by antigen was the same whether or not this antibiotic was present. Furthermore, in chambers prepared without antigen the cells migrated as well in concentrations of chloramphenicol up to 1000 μg/ml as in normal media. It is of interest that chloramphenicol in concentrations of 500 μg/ml or 50 μg/ml did not inhibit the incorporation of C^{14}-leucine into precipitable protein of peritoneal exudate cells when assayed over a 3½ hour period. Thus, in this system, no effect of chloramphenicol could be seen on the interaction of sensitive cells with antigen, or on protein synthesis in peritoneal cells. It should be noted, however, that marked inhibition of protein synthesis in intact mammalian cell systems may require treatment with chloramphenicol over several days prior to assay (10, 11).

TABLE IV

The Effect of Actinomycin D on the Migration of Sensitive Cells in the Presence of Antigen

Experiment	Migration in antigen			
	No actinomycin	10 μg	1 μg	0.1 μg
	per cent	*per cent*	*per cent*	*per cent*
1*	24		21	
2*	29	85	31	
3‡	61	116	64	62
4‡	9	105	60	17
5‡	7	50	37	
6‡	0	60	59	

* DNP-BGG–sensitive cells, 100 μg DNP-BGG/ml in chambers.

‡ Tuberculin-sensitive cells, 15 μg PPD/ml in chambers.

DISCUSSION

The migration of peritoneal exudate cells obtained from animals exhibiting delayed hypersensitivity is consistently inhibited by specific antigen. The present experiments indicate that this *in vitro* expression of delayed hypersensitivity is dependent upon active protein synthesis. In the presence of puromycin, a drug known to suppress protein synthesis, sensitive cells are no longer inhibited from migrating by antigen. The concentration of puromycin which prevents the effect of antigen on sensitive cells also inhibits the incorporation of C^{14}-leucine into peritoneal cell protein; the concentration of puromycin that has no

effect on the sensitive cell-antigen reaction does not inhibit the incorporation of labeled precursor into protein. Additional evidence that the action of puromycin in these studies is a result of its specific effect on protein synthesis was obtained with analogues of puromycin. Those structural analogues known to inhibit protein synthesis also prevent the action of antigen on sensitive cells while those which do not suppress protein synthesis have no effect.

Puromycin acts by effecting premature release of partially formed peptides from ribosomes and thus interferes with protein synthesis at a late stage (12). To determine whether the proteins involved in the sensitive cell-antigen reaction were directed by messenger RNA that was rapidly turning over, experiments were carried out using actinomycin D and chloramphenicol. It was observed that sensitive cells in the presence of actinomycin and antigen consistently migrated further than in antigen alone. Actinomycin also suppressed the uptake of C^{14}-uridine into acid precipitable material in peritoneal cells. Since it is known that actinomycin suppresses DNA dependent RNA synthesis (13), these results suggest that newly formed RNA is involved in this system. The possibility that actinomycin may exert effects on intact mammalian cells by mechanisms other than its suppressive action on RNA synthesis cannot be ruled out.

The present findings are consistent with results of previous studies using the cell-migration system which have indicated that actively metabolizing cells are necessary for the expression of delayed hypersensitivity *in vitro* (2, 3). They are also in accord with data obtained from other studies on the passive transfer of delayed hypersensitivity in guinea pigs (14, 15).

The abrupt suppression of protein synthesis brought about by puromycin must of necessity result in profound and widespread changes in cell function and metabolism. For example, pinocytosis can be suppressed in mouse peritoneal cells by inhibitors of protein synthesis, as Cohn has recently demonstrated (16). Thus a possible explanation for the action of puromycin in these experiments is that the drug, by inhibiting pinocytosis, prevents the entrance of antigen into the cells. It is of interest, in this context, that sensitive cells can be temporarily desensitized by trypsin; one of the mechanisms suggested to explain this effect was that receptor sites for antigen were removed from the cell surface by the enzyme (17).

There are numerous other cellular events which might suffer from the interruption of protein synthesis, ranging from the inhibition of specific immunoprotein synthesis to alterations of cell surfaces which may influence cell stickiness. The data at hand clearly demonstrate that the specific inhibition of sensitive cell migration by antigen requires protein synthesis; it does not, however, indicate in what manner protein synthesis is directly related to delayed hypersensitivity.

SUMMARY

Peritoneal cells from guinea pigs exhibiting delayed hypersensitivity are inhibited from migrating *in vitro* by specific antigen. This inhibition is prevented by the addition of puromycin to the culture medium. The amount of puromycin necessary to prevent the inhibition by antigen also suppressed the incorporation of C^{14}-leucine into peritoneal cell protein. Additional evidence that the action of puromycin is due to its inhibition of protein synthesis has been obtained with analogues of puromycin; those that inhibit protein synthesis also prevent the action of antigen on the cells, while those analogues that do not inhibit protein synthesis have no effect. Actinomycin also prevents the inhibition of sensitive cells by antigen while chloramphenicol has no effect. The data indicate that the inhibition of sensitive cell migration by antigen requires active protein synthesis. The possible mechanisms by which inhibition of protein synthesis may influence the *in vitro* reactions of delayed hypersensitivity are discussed.

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