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Intracellular immunoglobulin production *in vitro* by lymphocytes from patients with hypogammaglobulinaemia and their effect on normal lymphocytes

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SUMMARY

The ability of peripheral blood lymphocytes from twenty-two patients with late onset (acquired or common variable) hypogammaglobulinaemia to produce immunoglobulin was assessed by the immunofluorescent detection of intracytoplasmic immunoglobulin (Ic–Ig) in cultures stimulated with pokeweed mitogen (PWM). Intracellular immunoglobulin was found in 4·9–26% of cultured cells from eighteen out of nineteen controls. In contrast nineteen out of twenty-two patients with hypogammaglobulinaemia showed values less than 1% and in ten no Ic–Ig was detected. Two of the remaining three patients showed normal values. Lymphocytes from eleven patients showing less than 1% positive cells were selected for mixture experiments. Lymphocytes from five of the eleven patients strongly depressed immunoglobulin synthesis by normal lymphocytes when mixed together in the presence of PWM. However, lymphocytes from these individual patients did not depress immunoglobulin production in all normal controls.

INTRODUCTION

Wu, Lawton & Cooper (1973) found that three out of four patients with late onset hypogammaglobulinaemia, with virtually absent IgM and IgA and low IgG serum levels, developed intracellular immunoglobulin when cultured with PWM. In contrast Waldmann *et al.* (1974) found only one out of eleven patients produced intracellular immunoglobulin. For this reason a group of twenty-two patients with late onset hypogammaglobulinaemia were studied for their ability to produce intracellular immunoglobulin *in vitro*. Positive results were obtained in about 19% of patients.

Waldmann *et al.* (1974) also investigated the effect of immune deficient lymphocytes on the ability of normal lymphocytes to make intracellular immunoglobulin by cultivating them together in the presence of PWM. The present communication confirms their findings that immune deficient lymphocytes inhibit the immunoglobulin synthesis of normal lymphocytes. However, interpretation is complicated by the finding that the inhibitor effect depends upon the normal donor used.

MATERIALS AND METHODS

Patients and controls. The twenty-two patients aged between 11 and 65 (mean 34) years had late onset (primary acquired) hypogammaglobulinaemia with negative family histories. In only three was the onset

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before 2 years and sixteen were males. In all but one the serum IgA was below the limit of detection (i.e. less than 2 mg%). IgM was less than 10 mg% in twelve patients and all but eight had IgG levels less than 300 mg% (while on gamma-globulin treatment). Only two patients had a serum IgG greater than 400 mg%. All these patients were ambulant and most of them were in a good state of health. The nineteen controls (aged 22–80, mean 38, years) were sixteen healthy hospital staff and three elderly hospital inpatients with chronic bronchitis seen during an acute exacerbation.

Assays. These are described in detail elsewhere (Janossy, 1974). Lymphocytes were harvested from heparinized blood on a Ficoll–Triosil gradient, washed three times with L15 medium and resuspended in RPMI 1640 medium supplemented with 10% fresh autologous serum, glutamine, penicillin and streptomycin. Cultures of 0·25 ml were set up in duplicate in flat-bottomed Cooke microtitre plates with the lymphocytes at 1×10^6/ml. In the mixture experiments, the cultures were also at a final concentration of 1×10^6 lymphocytes/ml but half was from each donor. The serum was a 50:50 mixture of the corresponding autologous sera. Stimulated cultures received 8 μl of PWM although similar results were obtained in thirteen experiments with 3 μl and 24 μl of PWM. All cultures were incubated for 7 days at 37°C in 5% CO_2/air. At 4 days the cultures were supplemented with 0·1 ml of fresh medium with serum but no PWM. At 7 days the cultures were examined with an inverted microscope and proliferation assessed by the presence of large cells, colonies and multiple cell layers. The plates were then centrifuged three times at 300 ***g*** for 10 min (Mistral 6L, MSE), washing with PBS, pH 7·4, between each centrifugation.

Cytocentrifuge preparations were fixed in 95:5 ethanol–acetic acid and then directly stained with fluoresceinated sheep anti-whole human immunoglobulin antiserum (1/10 dilution) (Wellcome, England). After mounting they were examined first under phase with tungsten illumination and total intact cells counted (small cells and blast cells). The numbers of cells positive for Ic–Ig in the same field was then determined with incident illumination fluorescent optics (Zeiss Universal Fluorescence Microscope, HBO 200 w/4 lamp, BG 12 exciter filter, and a 50 barrier filter). The slides were examined blindly and a cell was regarded as positive if the cytoplasmic fluorescence was bright and there was a distinct nuclear shadow. In no case was background fluorescence a problem. Cultures which did not receive PWM did not show more than a very occasional Ic–Ig positive cell (< 0·025%).

RESULTS

Cells from nineteen normal individuals cultured for 7 days with PWM developed 1·4–26% of cells staining for Ic–Ig. The mean frequency of positive cells was 10·7%, only one person giving a very low number (1·4%). The values shown by twenty-two patients with hypogammaglobulinaemia contrasted strongly with those given by the normals. Nineteen patients showed a frequency of positive cells below 0·8%. Nine of these showed no positive cells while in ten there was a small but definite number of positive cells ranging from two in the range 0·6–0·8% and the remaining eight between 0·1–0·4%. The remaining three patients showed higher numbers of positive cells. Two of them showed 22·3% and 15% which was within the normal range while another patient showed 3%. The opportunity arose to test the two patients showing 15% and 3% positive cells again. This time there were 8% and 1·6% positive cells respectively and in both cases the cells were positive for IgM but not for IgG or IgA (using Wellcome fluoresceinated sheep anti-IgM, anti-IgG and anti-IgA antisera, 1/10 dilution).

To investigate the effect of cells from patients with hypogammaglobulinaemia on the ability of normal lymphocytes to produce immunoglobulin, lymphocytes from eleven patients producing less than 1% positive cells in response to PWM were selected and mixed with normal cells. In cell mixture experiments there is a problem as to the basis of comparison between cells cultured alone and cells cultured in a mixture. In the present experiments cells were cultured singly at 1×10^6/ml, and in mixtures $0{\cdot}5 \times 10^6$ of both types were present giving the same final concentration. The *expected percentage positive* in the mixture was the average of the figures in the single cultures. This usage was followed for mixtures of normal cells. In the mixture of immunodeficient and normal cells the immunodeficient cells made little or no contribution and their *expected percentage positive* was regarded as half the percentage of positive cells found in the normal lymphocytes alone. For convenience in Tables 1 and 2 this arithmetic is done by doubling the number of positives in the mixtures involving immunodeficient cells.

TABLE 1. Percentage cells positive for intracytoplasmic immunoglobulin following PWM stimulation

Experiment number	Control alone	Patient alone	Mixtures: Control + patient	Mixtures: Control + control
1	P.M. 9·2	F.R. 0·25	P.M.+F.R. 0·9 (1·8)*	P.M.+P.E. 11·1
	P.E. 9·9		P.E.+F.R. 5·6 (11·2)	
2	F.E. 8·0	B.R. 0·8	F.E.+B.R. 3·8 (7·6)	F.E.+E.M. 7·0
	E.M. 7·2		E.M.+B.R. 0·7 (1·4)	
		W.I. 0	F.E.+W.I. 1·8 (3·6)	
			E.M.+W.I. 0·3 (0·6)	
3	B.A. 7·6	L.E. 0	B.A.+L.E. 8·1 (16·2)	B.A.+O.L. 6·8
	O.L. 6·4		O.L.+L.E. 4·8 (9·6)	
4	B.M. 20·2	S.H. 0	BM+S.H. 2·2 (4·4)	B.M.+M.S. 31·6
	M.S. 12·4		M.S.+S.H. 1·0 (2·0)	B.M.+N.E. 14·6
	N.E. 17·3		N.E.+S.H. 4·6 (9·2)	B.M.+E.M. 10·5
	E.M. 13·1		E.M.+S.H. 9·4 (18·8)	M.S.+N.E. 27·7
				M.S.+E.M. 19·0
				N.E.+E.M. 16·4
5	P.U. 5·8	P.R. 0	P.U.+P.R. 7·6 (15·2)	n.d.
	M.P. 1·4	C.L. 0	P.U.+C.L. 11·2 (22·4)	
			M.P.+P.R. 2·2 (4·4)	
			M.P.+C.L. 6·4 (12·8)	
6	S.A.† 7	C.O. 0	S.A.+C.O. 0·5 (1·0)	S.A.+M.A. 8·5
	M.A.† 10·2	W.K. 0·6	M.A.+C.O. 0·2 (0·4)	
			S.A.+W.K. 4·6 (9·2)	
			M.A.+W.K. 3·6 (7·2)	
7	O.L. 9·6	C.A. 0	C.A.+C.L. 3·6 (7·2)	n.d.
8	J.A. 6·5	B.U. 0	J.A.+B.U. 4·2 (8·4)	n.d.
9	N.O. 5·4			N.O.+S.T. 12·6
	S.T.† 4·9			N.P.+R.E. 2·5
	R.E. 8·3			R.E.+S.T. 12·9

n.d. = Not determined.

* The figures in parentheses are the observed figures (not in parentheses) doubled, and are used for the analysis in Table 2.

† Hospital inpatients with chronic bronchitis.

When normal cells are mixed together in the presence of PWM the percentage of positive cells is usually the same or greater than expected on simple arithmetical grounds. In contrast when immunodeficient cells are mixed with normal cells the percentage is usually smaller than expected. The basic data are presented in Table 1.

For statistical purposes the mixture results are classified as $<5\%$, 5–10% and $>10\%$ cells positive for Ic–Ig. When the single cell cultures from single control individuals are compared with mixtures involving normal individuals it is seen (Table 2) that the mixtures show a greater percentage of positive cells than the single cultures ($P<0{\cdot}05$). In contrast the mixtures of normal and patient cells gave a smaller percentage of positive cells than expected ($P<0{\cdot}05$).

Overall, there is a clear inhibitory effect in mixtures of immunodeficient and normal cells. However, only five of the eleven patients showed strong inhibitory effects (F.R., B.R., W.I., S.H., S.M., C.O.; Table 1) and of the others W.K., C.A., B.U. showed little or no effect and two (P.R., C.L.) showed enhancement. It is interesting that within an experiment (expt no. 1, 2, 4) a patient may have an inhibitory effect on one control but not on another.

TABLE 2. Analysis of effects of lymphocyte mixtures intracytoplasmic immunoglobulin production

		< 5%	5–10%	> 10%	Total number of experiments
Number of:					
Controls alone (1×10^6/ml)		2 (11)*	12 (63)	5 (26)	19
Control + control ($0{\cdot}5\times10^6+0{\cdot}5\times10^6$) mixtures		1 (8)	3 (23)	9 (69)	13
Control + patient ($0{\cdot}5\times10^6+0{\cdot}5\times10^6$) mixtures	×2‡	9 (41)	7 (32)	6 (27)	22
Patients alone		11 (100)†	0	0	11

* Figures in parentheses are the percentage of total number of experiments.

† All patients below 1% positivity.

‡ All control/patient mixture data is doubled to allow for expected halving of results due to the unresponsive immunodeficient half of the mixture population.

Control + control mixtures significantly greater than controls alone:

$$\chi^2 = 6{\cdot}03, P< 0{\cdot}05.$$

(Control + patient mixtures) ×2 significantly less than controls alone:

$$\chi^2 = 5{\cdot}68, P< 0{\cdot}05.$$

DISCUSSION

The present results show that a few patients with late onset hypogammaglobulinaemia make appreciable amounts of Ic–Ig *in vitro* when stimulated with PWM. Half the remaining patients produce no detectable positive cells while the others showed between 0·1–0·4%. These figures are similar to those of Waldmann *et al.* (1974) who found one positive out of eleven patients in his immunodeficient group. They apparently differ from Wu *et al.* (1973) who found three out of four positive but this difference may reflect the small number of patients and, possibly, patient selection.

The finding of antigen-binding cells and cells with membrane-bound Ig in patients with late onset hypogammaglobulinaemia (Dwyer & Hosking, 1972) gave rise to the view that there is a block in the *in vivo* maturation of B cells in these patients. The observation that a few patients are able to make a normal percentage of immunoglobulin-containing cells *in vitro* shows that this defect can be overcome and pinpoints the problem in the *in vivo* maturation of immunoglobulin producing cells. Studies of patients with isolated IgA defects have also brought out the paradox of failure to produce IgA *in vivo* with normal numbers of IgA-containing cells following stimulation with PWM *in vitro* (Wu *et al.*, 1973).

It has been shown (Jacobson *et al.*, 1972b; Jacobson & Herzenberg, 1972a) that suppression of immunoglobulin production of a particular allotype was due to suppressor T cells. This raises the question of the role of suppressor cells in human immunodeficiency diseases. The present experiments show that mixtures of normal and immunodeficient cells contain on the average unduly few cells positive for Ic–Ig after stimulation with PWM. There are two main possibilities. One attractive view is that the immunodeficient lymphocytes contain suppressor cells (Waldmann *et al.*, 1974). An alternative view, in the light of the knowledge that products from T cells are required for immunoglobulin production by B cells (Schimpl & Wecker, 1972; Feldmann & Basten, 1972) is that B cells may remove these factors from

solution and hence immunodeficient cells might depress the performance of normal cells by utilizing T-cell factors and not by actively suppressing the normal B-cell response.

The fact that a patient's lymphocytes may depress Ic–Ig production by some but not all normal individuals requires analysis. One possibility is that when cells of certain HLA types are mixed the resulting allogeneic reaction gives rise to factors which favour Ic–Ig production (see Katz, 1972) or interfere with the action of the inhibiting effect. An alternative explanation is that, as in certain T–B cell interactions in mice, helper (and possibly suppressor) effects require that the interacting cells share certain histocompatibility complex surface antigens (Katz, 1974). A third possibility is that for physiological reasons cell populations vary in the ease with which their Ic–Ig synthesis can be inhibited. For instance, the outcome of a T–B cell interaction may depend upon the balance between helper and suppressor effects (Katz & Benacceraf, 1972; Gershon, Orbach-Arbouys & Calkins, 1974) and those normal individuals not inhibited by patients' cells might have a high level of helper effects. These considerations illustrate the problems which arise in interpreting results obtained from cell mixture experiments. Further advances in resolving the defects in hypogammaglobulinaemia at a cellular level are likely to require the use of purified cell populations and the search for soluble suppressor factors.

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