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Reversible Loss of Surface Receptors on Lymphocytes

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Summary. An α-2-glycoprotein from bovine serum (Fraction C) with immunosuppressive properties *in vivo* and anti-proliferative activity *in vitro* has been shown to reversibly inhibit the synthesis of cell surface receptors *in vitro*. The proliferative response of human lymphocytes *in vitro* to PHA and PPD can be inhibited by prolonged preincubation with Fraction C, which also inhibits the ability of lymphoid cells to bind PHA, 3–4 days for 90 per cent inhibition, and anti-immunoglobulin, 10 hours for 90 per cent inhibition. This inhibition is reversible and the binding ability recovers after removal of Fraction C at a similar rate to that at which it declined. Fraction C also rapidly and reversibly inhibits the secretion of antibody by immune spleen cells *in vitro*. The anti-proliferative effect of Fraction C is shown to involve an effect on ribosomal RNA synthesis and it is suggested that the mechanism of this action depends on the inhibition of protein synthesis, probably mediated by an effect on newly synthesized messenger RNA. The demonstration of different turnover rates of cell surface components on different types of cells suggests that it may be possible to use Fraction C to affect responsiveness of one type of lymphocyte while leaving another type unaffected.

INTRODUCTION

An α-2-glycoprotein from bovine serum, Fraction C, has been shown to inhibit the *in vitro* responsiveness of lymphocytes to a variety of stimuli (Milton, 1971) and to inhibit the immune response to sheep red cells *in vivo* when given 10–20 hours before the antigen (Mowbray, 1967). Early studies to be described here showed that Fraction C treatment of lymphocytes induced to proliferate *in vitro* with PHA caused a marked inhibition of ribosomal RNA synthesis, which was considered to be mediated via an effect on protein synthesis. To investigate whether the unresponsiveness of lymphocytes to stimuli when treated with Fraction C was due to an inhibitory effect on protein synthesis, leading to a loss of cell receptors for the stimuli, a study was undertaken of the following synthetic processes occurring in lymphoid cells:

1. The secretion of antibody by immune spleen cells.
2. The synthesis of receptors for PHA by blood or lymph node lymphocytes as measured by their ability to bind labelled PHA and to produce a proliferative response to PHA.
3. The synthesis of immunoglobulin on the surface of lymph node cells as measured by their ability to bind labelled anti-immunoglobulin.
4. The synthesis of receptors for PPD as measured by the ability of blood lymphocytes to produce a proliferative response to PPD.
5. Ribosomal RNA synthesis in PHA-stimulated blood lymphocytes.

MATERIAL AND METHODS

Antibody production by immune mouse spleen cells in vitro

Outbred Porton mice were immunized with 0·1 ml 10 per cent sheep red blood cells intravenously 5 days before removal of their spleens. The spleens were squashed with a rubber bung in Tris-buffered Eagles minimal essential medium (Milton, 1971) supplemented with 10 per cent foetal calf serum (Flow Laboratories Ltd) inactivated by heating at 56° for 4 hours. The fragments were allowed to settle and the dispersed cells were resuspended in bicarbonate buffered medium with 10 per cent foetal calf serum. Cultures were set up in Bijou bottles containing 2 ml medium and 10^7 nucleated cells, and either 0·4 ml Fraction C or 0·4 ml 0·9 per cent NaCl. The gaseous phase was 86 per cent nitrogen 10 per cent oxygen and 4 per cent carbon dioxide. Whole cultures were used for measurement of haemolytic plaques by the technique of Jerne.

Binding of anti-IgG to lymphocytes

Experiments in design similar to those for measuring the binding of PHA to lymphocytes were used, except that ^{125}Iodine-labelled rabbit anti-rat IgG was used instead of labelled PHA. The rabbit antibody was raised against rat IgG prepared by chromatography on DEAE cellulose. The antigen by immunoelectrophoresis contained only IgG2 and the antibody raised showed two lines against IgG2. By investigation with monospecific reagents the antiserum was shown to react with IgG2a and IgG2b, but not with IgG1. The IgG fraction of the rabbit antiserum was prepared by DEAE chromatography, the purity checked by immunoelectrophoresis and labelled with ^{125}Iodine. Before use the labelled antibody was made up in 10 per cent normal rabbit serum to diminish aggregation and centrifuged at 100,000 ***g*** for 20 minutes before it was used. The absorption by the cells was carried out at 4° for 30 minutes.

RESULTS

I. EFFECT OF FRACTION C ON ANTIBODY SECRETION BY IMMUNE SPLEEN CELLS

Cultures of immune mouse spleen cells always showed a decrease in the number of haemolytic plaques per culture with increasing duration of culture but when a dose of Fraction C twice that required to produce > 90 per cent inhibition of thymidine in a PHA stimulated culture was added to the cultures there was a greater decrease in plaque forming ability, Fig. 1. The number of plaques formed by Fraction C treated cultures is expressed in Fig 2 as a percentage of those formed by control cultures incubated for the same period. It was also noticed that the plaques formed by cells from the treated cultures were smaller than those in control cultures, particularly after the longer periods of incubation.

The amount of haemolytic antibody in the culture supernatant of Fraction C treated cultures was always less than that of control cultures and is expressed as percentage of the control antibody level in Fig. 2 because of the decrease in antibody in all cultures with increasing duration of culture. Table 1 shows that removal of Fraction C allowed production of haemolytic antibody to recover at a similar rate to that at which it declined.

II. EFFECT OF FRACTION C ON THE ABILITY OF LYMPHOCYTES TO BIND ANTI-IMMUNOGLOBULIN

When rat lymph node cells were incubated in the presence of Fraction C the ability to

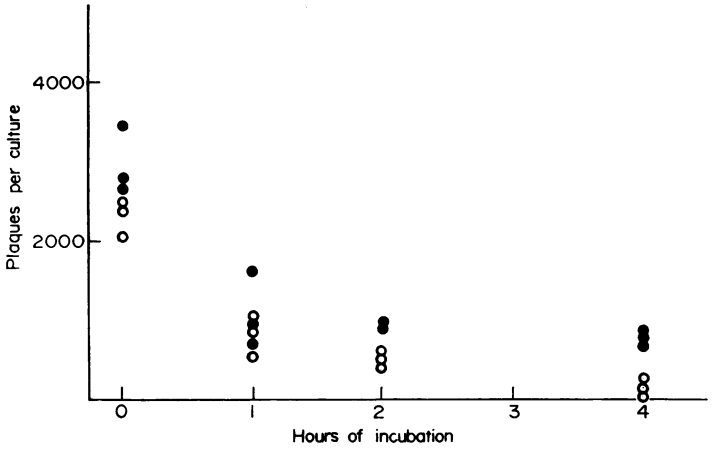

FIG 1. Effect of incubation with Fraction C on the direct plaque-forming ability against SRBC of immune mouse spleen cells. (●) 0·2 ml/ml 0·9 per cent NaCl. (○) 0·2 ml/ml Fraction C.

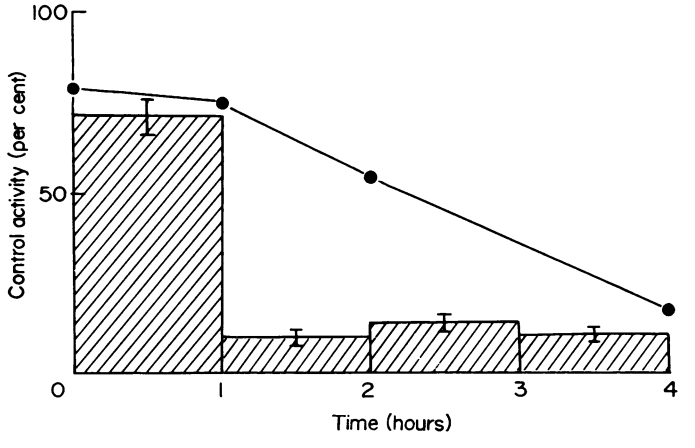

FIG. 2. Effect of Fraction C on antibody production against SRBC by immune mouse spleen cells. Results are expressed as a percentage of the antibody production in control cultures. (●) PFC per culture at time indicated. Histograms show the amount of haemolytic antibody released during a 1-hour period of incubation vertical bars show S.E.

TABLE 1
REVERSIBLE INHIBITION BY FRACTION C OF RELEASE OF HAEMOLYTIC ANTIBODY BY IMMUNE CELLS *in vitro*

Culture treatment		Antibody released (L_{50} units)	
0–2 hours	2–4 hours	2–3 hours	4–5 hours
Saline	Saline	—	1·78 ± 0·070
Fraction C	Saline	—	1·81 ± 0·045
Fraction C	Fraction C	—	0·35 ± 0·020
Fraction C	—	0·54 ± 0·020	—

Antibody production is expressed as L_{50} units/culture. One unit releases 50 per cent of the ^{51}Cr in the haemolytic assay.

bind rabbit anti-rat immunoglobulin decreased, Fig. 3, and when the cells were washed and resuspended in fresh medium the binding ability recovered at the same rate as that at which it had fallen.

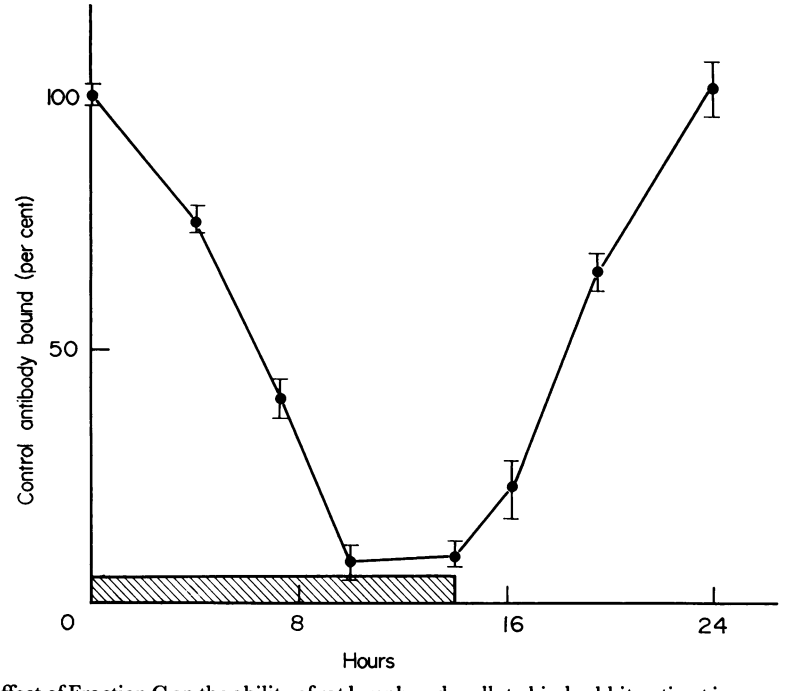

FIG. 3. Effect of Fraction C on the ability of rat lymph node cells to bind rabbit anti-rat immunoglobulin. Fraction C was present for the period indicated by the hatched area. Results are expressed as the percentage of the anti-immunoglobulin bound in control (saline-treated) cultures ± the sum of the standard errors of the control and treated cultures.

III. EFFECT OF FRACTION C ON THE ABILITY OF LYMPHOCYTES TO RESPOND TO PPD

When human peripheral blood lymphocytes were incubated with Fraction C at a dose of 0·1 ml/ml of culture prior to washing and resuspending in fresh medium it was found that 2 days preincubation were required completely to suppress the responsiveness to PPD, Fig. 4.

IV. EFFECT OF FRACTION C ON THE ABILITY OF LYMPHOCYTES TO RESPOND TO AND TO BIND PHA

When human peripheral blood lymphocytes were incubated with Fraction C prior to stimulation with PHA 4 to 5 days of preincubation were required to suppress the proliferative response, Fig. 5. When rat lymph node cells were incubated in the presence of Fraction C their ability to bind lentil PHA decreased to a few percent of the original binding capacity over 3 days and when the cells were resuspended in fresh medium the binding ability recovered in about 3 days, Fig. 5.

V. EFFECT OF FRACTION C ON DNA, RNA AND PROTEIN SYNTHESIS IN LYMPHOCYTES STIMULATED BY PHA

When lymphocytes cultured with PHA were treated with a dose of Fraction C that

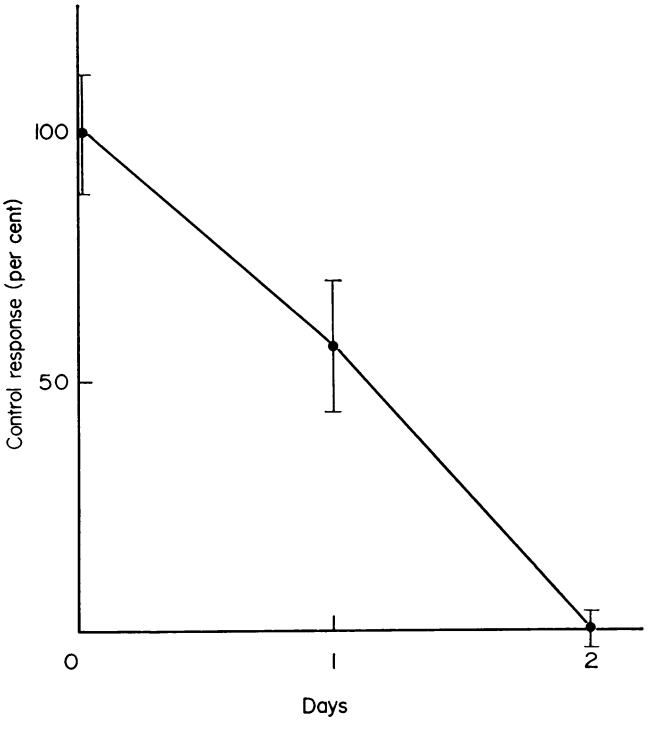

FIG. 4. Effect of preincubation with Fraction C on the subsequent ability of human peripheral blood lymphocytes to respond to PPD. The results show the proliferative response of lymphocytes to PPD after preincubation with Fraction C, expressed as a percentage of the response of control cultures preincubated for the same period. Results are expressed as:

$$\frac{\text{Treated culture–unstimulated culture}}{\text{Control culture–unstimulated culture}} \times 100 \text{ per cent.}$$

The vertical bars show the sum of the standard errors.

produced >90 per cent inhibition of DNA synthesis the effect on RNA and protein synthesis was much less, Table 2. It was also found that cells which had transformed to the stage where DNA synthesis was taking place were not affected in their ability to incorporate uridine or leucine by Fraction C treatment over a subsequent 6-hour period. Density gradient studies showed that the inhibitory effect of Fraction C on RNA synthesis in a PHA culture 24 hours after initiation was due to an effect on ribosomal RNA synthesis, Fig. 6. Fraction C added to a culture only for the 4-hour duration of the incubation with [^{3}H] uridine did not affect the RNA density gradient profile compared to control stimulated cultures. When Fraction C was added for 18 hours to a PHA culture that had been labelled with [^{3}H]uridine for the first 6 hours after initiation there was no release of labelled material from the cells, indicating that Fraction C affects RNA synthesis rather than RNA already present in the cells.

To measure the release of haemolytic antibody and the incorporation of [^{3}H]leucine into cell constituents cultures were centrifuged and the cells resuspended in fresh medium to which was added 4 μCi 4,5-[^{3}H]L-leucine and incubation was continued for a further hour. Then the cultures were centrifuged and the cells collected for radioactivity measurement by

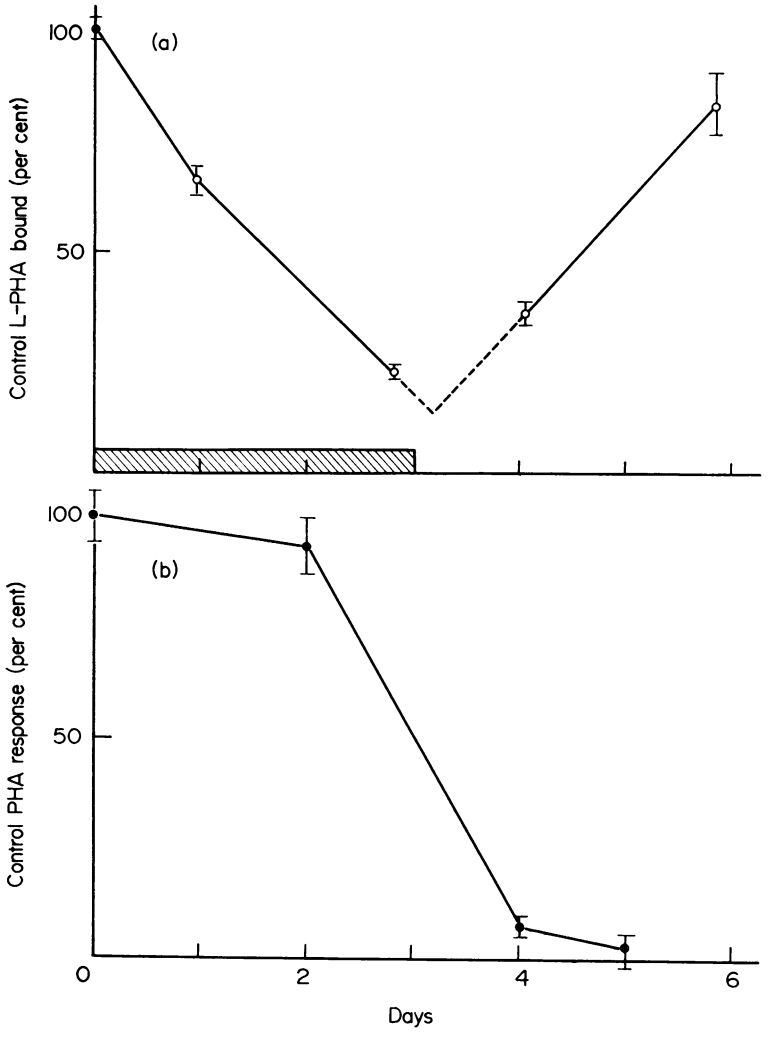

FIG. 5. (a) Effect of incubation with Fraction C on the ability of rat lymphocytes to bind lentil haemagglutinin. Binding of L-PHA is expressed as a percentage of that bound by control cultures incubated for the same period. Vertical bars show the sum of the standard errors. The hatched area indicates the duration of incubation with Fraction C. (b) Effect of preincubation with Fraction C on the subsequent ability of human blood lymphocytes to respond to PHA. The response to PHA is expressed as the counts incorporated in preincubated cultures as a percentage of those incorporated by control cultures preincubated for the same period. Vertical bars show the sum of the standard errors.

filtration through glass fibre discs (Milton, 1971) and the supernatants assayed for their content of haemolytic antibody for sheep red blood cells. Duplicate 2-ml samples of supernatant were added to 0·2 ml ^{51}Cr-labelled 2 per cent SRBC. After incubation for 30 minutes at 37° the tubes were centrifuged and the supernatants discarded. To the cell pellet was added 0·2 ml of a 2 per cent solution of fresh guinea-pig serum in barbitone buffered isotonic saline containing 5 mM calcium and 5 mM magnesium and the tubes were incubated for 15 minutes at 37°. Then 1 ml buffered saline was added, the

TABLE 2

EFFECT OF FRACTION C ON DNA, RNA AND PROTEIN SYNTHESIS IN CULTURES OF HUMAN LYMPHOCYTES STIMULATED BY PHA

	Incorporation (cpm)					
	Uridine (hours)				Leucine (hours)	Thymidine (hours)
	4	8	20	48	48	48
Unstimulated	590	610	690	810	150	260
PHA	690	1440	5080	9300	1680	15390
PHA Fr C	510	860	2730	4270	530	560

Two-millilitre cultures contained 1×10^6 lymphocytes and either 0·2 ml of Fraction C or 0·2 ml of 0·9 per cent NaCl was added at the beginning of the culture period. Tritium labelled compounds were added for the last hour of culture for uridine and thymidine and for the last 2 hours for leucine. The time shown is the time of termination of the culture. The counts shown are the mean of triplicate cultures which did not vary by more than ± 15 per cent.

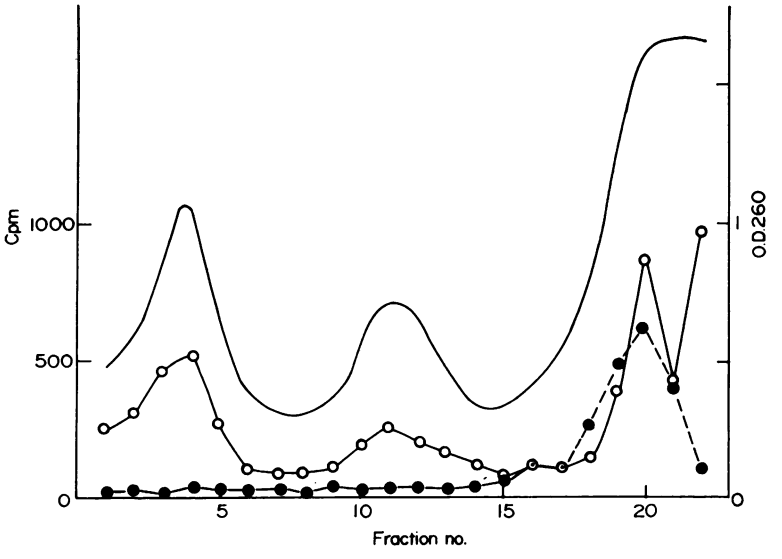

FIG. 6. Effect of Fraction C on RNA synthesis in cultures of human lymphocytes stimulated by PHA. 10-ml cultures contained 10^7 lymphocytes, 100 µl PHA and 1 ml Fraction C or 0·9 per cent NaCl. 20 µCi [^{3}H]uridine were added after 20 hours of culture and cultures were harvested at 24 hours. The continuous line shows the O.D. at 260 nM of the gradient fractions. (○) Cpm per fraction—control PHA culture. (●) Cpm per fraction—Fraction C treated PHA culture.

tubes agitated and centrifuged, the radioactivity of the decanted supernatant measured and the percentage lysis calculated.

Culture of human lymphocytes for stimulation by PHA and by PPD

Human lymphocyte cultures were set up as previously described (Milton, 1971). For the measurement of RNA synthesis 2 µCi 5-[^{3}H]Uridine was added to a 2-ml culture for the last hour of incubation and for measurement of protein synthesis 4 µCi 4,5-[^{3}H]L-leucine was added for the last 2 hours of incubation. Cells were harvested on glass fibre discs and

radioactivity measured as previously described (Milton, 1971). In those experiments involving preincubation with Fraction C before stimulation by PHA or PPD cell counts done on cultures after resuspension in fresh medium showed that in no case was there a loss of more than 10 per cent of cells in treated compared to control cultures. Viable cell counts done by the trypan blue dye exclusion test, 0·1 ml 1 per cent trypan blue per 2 ml cell suspension, never showed more than 5 per cent stained cells and so the total cell count was taken to represent an accurate measure of viable cells present.

Density gradient analysis of lymphocyte RNA synthesis

Human peripheral blood lymphocyte cultures were set up in universal bottles containing 10^7 cells in 10 ml medium, 0·1 ml PHA and, 1 ml Fraction C or 0·9 per cent NaCl. Cultures were terminated at 24 hours. For the last 4 hours of culture 20μ Ci [^{3}H]uridine were added. At the end of the culture period 1 ml of cell suspension was removed for harvesting on a glass fibre disc. The remainder of the culture was used for extraction of the RNA for density gradient analysis using fresh rat liver post-mitochondrial supernatant as carrier (Kay and Korner, 1966). The cultures were centrifuged, resuspended in 0·5 ml 0·1 M acetate buffer pH 5·0 containing 0·1 per cent sodium dodecyl sulphate (SDS) and 7·5 ml phenol saturated with acetate buffer with SDS was added. To this was added 2 ml post-mitochondrial supernatant. The phenol extraction was repeated and the aqueous RNA solution was washed three times in ether, the remaining ether being blown off by passing air through the solution, which was then added to 8 volumes of methanol. The precipitate of RNA was allowed to form at $-20°$ for 30 minutes after which it was centrifuged, dissolved in 0·4 ml acetate buffer with SDS and layered on a gradient of 5–25 per cent sucrose in acetate buffer with SDS. The gradient was run for 150 minutes at 50,000 rev/min, 190,000 *g* at average radius, in an MSE-65 ultracentrifuge at 5–8°. Gradient fractions were diluted to 2 ml with acetate buffer and the O.D. at 260 nm measured. The incorporation of tritium into the fractions of the gradient was measured by adding 5 mg yeast RNA (0·25 ml 2 per cent solution) to the diluted gradient fractions and then precipitating the RNA with 8 volumes of methanol. The precipitate was collected on a glass fibre disc and the radioactivity measured.

Binding of phytohaemagglutinin to lymphocytes

Because of its ease of purification *Lens culinaris* PHA was used in this part of the study, prepared from lentil beans after the method of Howard, Sage, Stein, Young, Leon and Dyckes (1971) and purified by absorption to Sephadex G-200 and elution with 0·1 M glucose. The preparation was concentrated and dialysed against 0·1 M NaCl, after which it was assayed for its mitogenic potential and haemaggutinating activity, using rat cells. The active preparation was iodinated with ^{125}Iodine by the technique of MacFarlane (1958).

Rat peripheral lymph node cells were placed in culture as described above, with or without Fraction C in the medium. At intervals after starting the incubation radioactive PHA was added to triplicate cultures which were then left for 30 minutes at 4° after which the cells were washed three times and the bound radioactivity measured. From previous experiments an amount of PHA was used of which 75 per cent bound to the cells present in a 2-ml culture. From Scatcherd plots the average number of binding sites per cell was calculated for normal and treated cells by the method of Steck and Wallach (1965) and all results expressed as the percentage of the number of sites binding PHA on control incubated untreated cells.

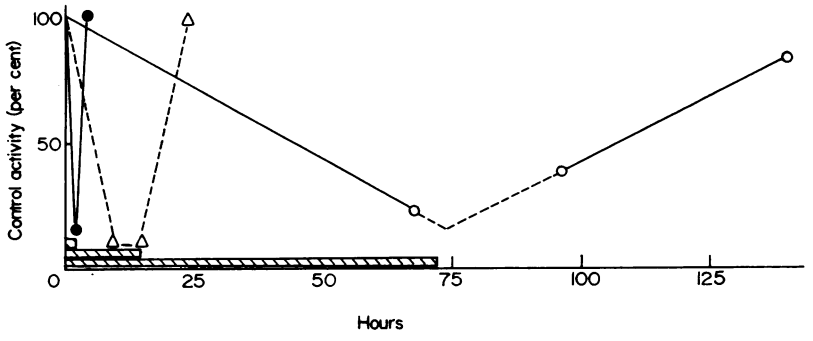

FIG. 7. Time course of the inhibition by Fraction C and the recovery therefrom of various synthetic activities of lymphoid cells. (●) Secretion of haemolytic antibody. (△) Binding of anti-immunoglobulin. (○) Binding of L-PHA. Fraction C was present in incubations for the period indicated by the hatched areas.

DISCUSSION

Previous experiments have shown that Fraction C is most effective at suppressing the immune response of mice to sheep red blood cells when given 10–20 hours before antigen (Mowbray, 1967) and this can now be explained by the loss of immunoglobulin on the surface of lymphocytes, presumably B cells, which is here shown to require about 10 hours treatment with Fraction C *in vitro*. The loss of ability to respond to PPD *in vitro* requires a longer period of treatment with Fraction C which is probably because the responding cells are predominantly T cells.

The loss of ability to bind PHA is much slower which might perhaps be expected for a structural cell component rather than a functional one. The ability to bind PHA and the ability to respond to PHA have an approximately parallel time course in so much as it takes about 4 days greatly to inhibit responsiveness, 93 per cent and 3 days to produce almost complete loss of binding ability, 85 per cent.

That lymphocytes can still respond normally to PHA after 48 hours preincubation with Fraction C indicates that the cells are not generally incapacitated as regards their proliferative ability, which is good evidence that their lack of responsiveness to PPD is due to a specific alteration in their ability to respond to antigenic stimuli. It is likely that this is due to loss of a specific receptor protein.

The experiments on plaque formation and release of haemolytic antibody show a rapid inhibition of protein production which is reversible. The observation that there is no inhibition of total leucine incorporation suggests that most of the protein synthesis occurring in the cells is controlled by longer lived messenger RNA than that involved in the synthesis of a secreted protein exemplified by immunoglobulin. It is also possible that non-functional protein is being synthesized.

The lack of effect of Fraction C on thymidine, uridine and leucine incorporation in a transformed lymphocyte culture is consistent with the proposition that messenger RNA is affected, which is likely not to have any immediate effect on protein synthesis as measured by total leucine incorporation. Also it suggests that DNA synthesis continues over a 6-hour period utilizing enzymes or messenger RNA already present in the cell.

The observation of inhibition of ribosomal RNA synthesis in cultures treated for 24 hours with Fraction C, whereas there is no effect on ribosomal RNA synthesis in cultures treated for 4 hours, suggests strongly that there is no direct effect on ribosomal RNA

precursors but that the effect is exerted via an effect on protein synthesis comparable to the inhibitory effect of cycloheximide or ribosomal RNA synthesis in lymphocytes transformed by PHA (Kay and Korner, 1966; Cooper, 1971). Although only partial inhibition of RNA and protein synthesis is observed in Fraction C treated PHA cultures complete inhibition of DNA synthesis is likely as protein requirements for DNA synthesis are rigorous.

Since therefore there is strong indication that specific protein synthesis is inhibited by Fraction C in stimulated lymphocytes the different time course of the effects on specific lymphoid cell proteins can be explained by different rates of turnover of the proteins or of their messenger RNA. A rapid effect is exerted on a secreted protein, haemolytic antibody, and slower effects are found with two different non-secreted proteins, immunoglobulin and PPD receptors which probably occur on different types of cells. An even slower turnover time is found for a structural cell component the receptor for PHA, Fig. 7. The final piece of evidence that the effect of Fraction C is to switch off specific protein synthesis is that the rate of recovery of detectable protein activity is the same as that at which it disappears.

As regards the mechanism of action of the inhibitory effect of Fraction C on protein synthesis the observation that it does not cause a rapid inhibition of overall protein synthesis would suggest that the effect is exerted at an earlier stage in the procession of information transfer from DNA to protein than the actual synthesis of protein. As Fraction C and similar immunosuppressive agents (Mowbray, 1967, Carpenter, Milton, Mowbray and Butterworth, 1972) all have ribonuclease activity associated with them we suggest that the initial effect is on the synthesis of the messenger RNA required for protein synthesis.

In conclusion it is suggested that IgG on B cells is turned over in about 10 hours, PPD receptor (antibody) on T cells in about 48 hours and PHA receptors on T cells in about 100 hours. It seems that Fraction C treatment is a useful approach to the study of turnover of lymphocyte components and it is possible to use it to make one type of lymphocyte unresponsive while leaving others intact.

ACKNOWLEDGMENTS

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