Serum Factors Affecting the Incorporation of [³H]Uridine by Lymphocytes Stimulated by Concanavalin A

STUDIES OF THE ROLE OF COMPLEMENT

By P. MILTHORP and D. R. FORSDYKE

Department of Biochemistry, Queen's University, Kingston, Ont., Canada

(Received 29 August 1972)

1. Rat lymph-node cells cultured in serum and medium 199 were activated to transform and proliferate by concanavalin A. Initial cell activation was assessed by measuring the enhanced radioactive labelling of cells with [³H]uridine produced by concanavalin A during the first 6 h of culture. 2. In medium containing serum the degree of activation was dependent on the ratio of concanavalin A to non-diffusible serum macromolecules; however, cells could be activated to a normal extent in a medium containing only diffusible molecules. This indicates that certain serum macromolecules buffer cell receptor sites against reaction with concanavalin A. 3. At high concanavalin A concentrations labelling was depressed below that of control cultures without concanavalin A. This inhibition of labelling (i) occurred in calf serum, but not in homologous serum, (ii) was removed by pretreatment of serum with various complement inhibitors, and (iii) first appeared after 1 h of culture following an initial phase of cell activation by concanavalin A. Cells pre-labelled with [³H]uridine slowly released the label into the culture medium; this rate of release was suddenly accelerated after 1 h of culture with concanavalin A if complement was present. The results suggest that inhibition of labelling requires the sequential binding of concanavalin A and then complement to the cell surface. 4. Results from experiments in which calf serum was mixed in various proportions with calf serum which had been preheated to inactivate complement, suggest (i) a requirement for complement in stoichiometric quantities dependent on the number of cells being inhibited, and (ii) that preheated serum can inactivate complement in unheated serum. 5. The proliferative response over 3 days of culture was assessed by measuring the enhanced labelling of cells with [³H]-thymidine produced by concanavalin A. In preheated calf serum two types of inhibition were noted. (i) A progressive inhibition at high concanavalin A concentrations so that the optimum response was shifted to lower concanavalin A concentrations as the duration of culture was extended; it is suggested that this reflects the secretion of complement by cultured cells. (ii) An inhibition of the optimum response appearing late in the culture period at high cell concentrations; it is suggested that this is due to the exhaustion of medium nutrients in most actively growing cultures.

When activated by the 'antigen analogues' phytohaemagglutinin and concanavalin A, cultured lymphocytes enlarge and transform within 2–3 days into large pyronine-staining cells which divide (Nowell, 1960; Marshall & Roberts, 1965; Milthorp & Forsdyke, 1970*b*). Enhanced labelling of the cells with radioactive RNA precursors is detectable in the first few hours of culture, and after 1 day DNA synthesis is initiated and morphological signs of transformation become evident. Previous studies have shown that a variety of serum factors, including complement and α -macroglobulin, may modify the lymphocyte response to various concentrations of these analogues (Forsdyke, 1966, 1967, 1968; Havemann *et al.*, 1970*a,b*; Milthorp & Forsdyke, 1970*a,b*; Walker & Lucas, 1971). The present paper presents an extension

of these studies with particular reference to concanavalin A.

The paper is in three parts. First, there is an examination of the effects of serum on the response to low concentrations of concanavalin A. Then, there is a detailed examination of the mediation by serum of the inhibition of the response at high concanavalin A concentrations. Finally, changing patterns of concanavalin A dose-response curves during prolonged incubation are described; a distinction is made between changes caused by ecological factors, such as the exhaustion of nutrients in the culture medium, and changes caused by other factors, such as the secretion of complement by the cultured cells. In the first two parts of the paper the response to concanavalin A is detected in the early hours of culture by

using [^3H]uridine. The main variables affecting the incorporation of this compound in the system were reported previously (Forsdyke, 1971a). In the third part of the paper [^3H]thymidine is used.

The results are thought to be relevant to the understanding of the normal mechanisms of the homeostatic regulation of cell population size *in vivo*. This is considered more fully in another paper in the context of the regulation of the size of a lymphocyte clone by the concentration of specific antigen (Forsdyke, 1971b). A preliminary report of this work has appeared (Milthorp & Forsdyke, 1972).

Materials and Methods

Details of materials and methods have been described previously (Milthorp & Forsdyke, 1970b; Forsdyke, 1971a). Female Wistar rats (150–200g) were obtained from Woodlyn Farms Ltd., Guelph, Ont., Canada. Medium 199 was obtained from the Grand Island Biological Co., Grand Island, N.Y., U.S.A. Concanavalin A, prepared by the procedure of Agrawal & Goldstein (1967), was obtained from Calbiochem, Los Angeles, Calif., U.S.A. The powder was dissolved in sterile water (50mg/ml) and samples (1 ml) were stored frozen at -20°C . No loss of activity was detected after storage in this manner for 12 months.

Serum

Homologous serum was prepared from 3–4 rats on the day of each experiment. Calf serum was obtained from Woodlyn Farms Ltd., Guelph, Ont., Canada, and samples (10ml) were stored at -20°C . On the day of initiating cell cultures, serum samples were treated in various ways designed to remove complement activity (Rapp & Borsos, 1970). These were (i) heating at 56°C for 30min followed by rapid cooling to room temperature, and (ii) incubation for 2h at 37°C with either inulin (10–20mg/ml of serum; British Drug Houses, Poole, Dorset, U.K.), or a yeast cell-wall preparation (Zymosan; 12–15mg/ml of serum; Calbiochem) or purified cobra-venom complement inhibitor (12 μg /ml of serum) kindly given by Dr. O. Götze and Dr. H. J. Müller-Eberhard, (Götze & Müller-Eberhard, 1971). The Zymosan and inulin were heated to 100°C for 10min in 0.14M-NaCl shortly before addition to serum. Zymosan particles were removed from serum by centrifugation (1200g, 10min), but inulin and cobra-venom inhibitor were not removed.

Cell culture

Rat lymph-node cells prepared from 1–2 rats were suspended in medium 199 and added to master culture vessels containing serum (20%, v/v), medium 199 and [$^5\text{-}^3\text{H}$]uridine (1 μCi /ml; 20–29Ci/mmol) at 37°C .

Samples (1ml) were added to round-bottomed glass centrifuge tubes containing various dilutions of concanavalin A in 20–50 μl volumes. The cultures were incubated in a humidified atmosphere of air+ CO_2 (95:5) at 37°C . At appropriate times cells were pelleted by centrifugation (1200g, 4min), washed in 0.14M-NaCl (4°C), recentrifuged, and finally stored (-20°C) in ethanol until determination of acid-precipitable radioactivity by using Hyamine (0.4ml) and a toluene-based scintillation 'cocktail' (10ml) containing 2,5-diphenyloxazole (5g/l). Counting efficiency at ambient temperature was approx. 32%. To study the proliferative response to concanavalin A during prolonged incubation, cultures were set up without [^3H]uridine and instead [^3H]thymidine (1 μCi /ml; 15–28Ci/mmol) was added 6–20h before the cells were harvested. To determine the radioactivity in the culture medium, 0.1 ml of the medium was added to 10ml of a 1,4-dioxan-based scintillation 'cocktail' containing naphthalene (100g/l) and 2,5-diphenyloxazole (5g/l). Counting efficiency was approx. 45%.

Results and Discussion

Dependence of response on ratio of concanavalin A to serum macromolecules

As in the case of phytohaemagglutinin (Forsdyke, 1967, 1968), the response to concanavalin A is dependent on the ratio of concanavalin A to serum (Milthorp & Forsdyke, 1970b). The macromolecular nature of the serum activity concerned was shown by adding to cultures the diffusate or dialysis residue from rat serum dialysed against an equal volume of medium 199 (Fig. 1). Whereas the diffusate did not influence the response, the dialysis residue shifted the ascending limb of concanavalin A dose-response curves to the right (Fig. 1a). When the concanavalin A concentration was expressed, not as μg of concanavalin A/ml of culture medium (Fig. 1a), but as μg of concanavalin A/ml of serum macromolecules present in cultures (Fig. 1b), the ascending limbs of dose-response curves became superimposable.

Thus the response is dependent on the ratio of concanavalin A to serum macromolecules. This is not surprising since concanavalin A, like phytohaemagglutinin, is known to react with a variety of serum macromolecules including immunoglobulin M and α -macroglobulin (Harris & Robson, 1963; Morse, 1968). Whether the activation of cells requires a critical reaction of concanavalin A with serum macromolecules or whether the macromolecules simply competitively buffer cells against reaction with concanavalin A, was then examined.

Activation by concanavalin A in serum diffusate

Beckman (1962) proposed that phytohaemagglutinin activates cells by removing a macromolecular

Fig. 1. Effect of the diffusate or dialysis residue from rat serum dialysed against an equal volume of medium 199, on the ascending limb of concanavalin A dose-response curves

Rat lymph-node cells ($1.1 \times 10^6/\text{ml}$) were incubated (5 h, 37°C) with [^3H]uridine ($1 \mu\text{Ci}/\text{ml}$; $30 \text{ Ci}/\text{mmol}$) and various concentrations of concanavalin A in medium containing rat serum (15%, v/v; ●), or rat serum (15%) supplemented with either the diffusate (30%; □) or dialysis residue (30%; ■) from homologous serum dialysed for 18 h at 4°C against an equal volume of medium 199. The total culture volume was made up to 1 ml with medium 199. In Fig. 1(a) the concanavalin A concentration is expressed as $\mu\text{g}/\text{ml}$ of medium. Fig. 1(b) shows the same results with the concanavalin A concentration expressed as $\mu\text{g}/\text{ml}$ of non-diffusible serum macromolecules present in culture. In Fig. 1(a) individual radioactivity values (c.p.m.) are plotted (duplicates). In Fig. 1(b) the mean value of duplicate cultures is plotted.

serum inhibitor of lymphocyte proliferation. In a previous study it was shown that the pool-corrected rate of labelling with [^3H]uridine of lymph-node cells was not increased by removing macromolecules from the culture medium; this suggested that a macromolecular inhibitor of proliferation was not involved (Forsdyke, 1971a). However, it is possible that the rather drastic procedure of removing all serum macromolecules had removed other cofactors required for lymphocyte activation. To study this further the response to various concentrations of concanavalin A was studied in culture medium containing medium 199 and either serum diffusate or serum dialysis residue (Fig. 2). In serum diffusate the cells responded at very low concanavalin A concentrations, as would be expected in a medium in which cell buffering by macromolecules against reaction with concanavalin A was decreased. The magnitude of the response was of the same order as that obtained in serum dialysis residue indicating that no macromolecular cofactors are required for activation to occur. These results could be most simply explained if a direct reaction of

concanavalin A molecules with cellular receptor sites (Allan *et al.*, 1972) were the sole requirement for cell activation to occur.

Complement-dependent inhibition of lymphocytes at high concanavalin A concentrations

In previous studies the labelling with [^3H]uridine of rat lymphocytes was enhanced by low concentrations of concanavalin A when either homologous serum or calf serum was used. At high concanavalin A concentrations labelling was inhibited. This inhibition was a function not only of the concanavalin A concentration but also of the serum since the inhibition only appeared in calf serum and not in homologous serum. The labelling was depressed below that of control cultures without concanavalin A indicating that the inhibition was more than just a failure of cells to respond to concanavalin A. The inhibition was removed by pretreating the calf serum in ways designed to inactivate serum complement (i.e. heating at 56°C for 30 min and incubating with Zymosan;

Fig. 2. Activation by concanavalin A of lymphocytes cultured in serum diffusate or serum dialysis residue

Rat lymph-node cells ($1.8 \times 10^6/\text{ml}$) were incubated (5h, 37°C) with [^3H]uridine ($1 \mu\text{Ci}/\text{ml}$; $24 \text{ Ci}/\text{mmol}$) and fourfold dilutions of concanavalin A in medium containing either the diffusate (13%; □) or the dialysis residue (13%; ■) from homologous serum dialysed against an equal volume of medium 199. The total culture volume was made up to 1 ml with medium 199. Individual radioactivity values (duplicates) are plotted.

Milthorp & Forsdyke, 1970a,b). To further characterize the serum activity the effects of other complement inhibitors were examined.

Fig. 3(a) shows that whereas preheating homologous serum had no influence on concanavalin A dose-response curves, preheating calf serum had two effects. (i) The ascending limb of the curves was shifted to the right, suggesting that the concanavalin A-binding capacity of the serum had been increased. (ii) The inhibition at high concanavalin A concentrations was removed so that the maximum response was of the same order as that obtained with homologous serum. The inhibition could also be partially removed by treating serum with inulin (Fig. 3a), in our hands a relatively poor complement inhibitor (D. R. Forsdyke, unpublished work), and completely removed by treating serum with Zymosan or with purified cobra-venom complement inhibitor (Fig. 3b). On the basis of these criteria at least one of the activities, and perhaps the only activity, in calf serum necessary for the inhibition may be identified as complement. In addition to its haemolytic powers,

complement may also lyse nucleated cells. Thus it is likely that inhibition at high concanavalin A concentrations is not caused directly by concanavalin A, but is mediated by serum complement. Further evidence in this respect was obtained in the experiments described below, which could be interpreted most simply with the assumption that the mediator was complement.

Effects of various proportions of heated and unheated calf serum

The extent of the inhibition at high concanavalin A concentrations varies with the batch of calf serum employed and can also vary with the serum concentration, while the ratio of concanavalin A to serum is kept constant (Milthorp & Forsdyke, 1970b). This was further investigated by mixing various proportions of heated and unheated serum, while the total serum concentration in cultures was kept constant. Fig. 4 shows results obtained at high concanavalin A concentrations with three batches of calf serum that differed in their ability to mediate inhibition. The most inhibitory serum (Fig. 4a) depressed the labelling far below that of control cultures without concanavalin A, whereas the least inhibitory serum (Fig. 4c) depressed the labelling so that it was similar to that in control cultures without concanavalin A. In control cultures only a very slight increase in labelling is produced by preheating serum (Milthorp & Forsdyke, 1970b).

In the presence of concanavalin A the curves describing the increase of labelling with increasing proportions of heated serum (decreasing proportions of unheated serum) were often of sigmoid appearance. For the most inhibitory serum (Fig. 4a) increasing the proportion of heated serum initially had no effect on labelling which suggested that this serum contained an excess of complement; only after a considerable decrease in the proportion of unheated serum could an enhancement of labelling be detected. In contrast, for the least inhibitory serum (Fig. 4c), labelling was enhanced by a small decrease in the proportion of unheated serum suggesting that complement was present at limiting concentrations. In such a serum, the degree of inhibition should be increased by increasing the serum concentration (Milthorp & Forsdyke, 1970b).

The maximum enhancement of labelling could occur in the presence of low proportions of unheated serum and did not require 100% heated serum (Fig. 4). This indicates that preheating serum, besides inactivating certain complement components, also confers on serum the ability to inactivate complement in unheated serum.

Whereas in most haemolytic systems the complement concentration soon limits the degree of haemolysis (Rapp & Borsos, 1970), in the present system

Fig. 3. Effect of pretreatment of calf serum with various complement inhibitors on the response to concanavalin A of rat lymphocytes cultured in the serum

In Fig. 3(a) rat lymph-node cells (1.3×10^6 /ml) were incubated (6h, 37°C) with [^3H]uridine ($1 \mu\text{Ci}/\text{ml}$; $23.3 \text{ Ci}/\text{mmol}$) in calf serum (20%, v/v) which had either been incubated at 37°C for 2h (○), or preheated at 56°C for 30 min followed by incubation at 37°C for 1.5 h (●), or treated with inulin (20mg/ml of serum for 2h at 37°C ; ▼). ----, Dose-response curves obtained with either preheated (56°C , 30 min) or unheated homologous serum (20%). Each point is the mean of duplicate cultures. Fig. 3(b) shows results from a separate experiment in which cells were incubated similarly in serum pretreated (2h, 37°C) either with Zymosan (12mg/ml of serum; ■) or cobra-venom complement inhibitor ($12 \mu\text{g}/\text{ml}$ of serum; ▲). The Zymosan particles were removed by centrifugation from the serum before use of the serum in culture, but inulin and cobra-venom inhibitor were not removed. ●, Serum preheated at 56°C for 30 min and then 37°C for 1.5 h; ○, serum incubated at 37°C for 2h.

complement appeared to be in excess (Figs. 4a and 4b). This is best understood in terms of the stoichiometric requirement of complement depending on the number of cells being attacked. In haemolytic systems there are generally of the order of 10^8 erythrocytes, whereas in the present system there were around 10^6 lymphocytes. Since the depressed labelling produced by concanavalin A and complement sometimes approached zero (Fig. 4a), it is likely that a high proportion of the 10^6 lymphocytes were capable of being attacked. There is no reason to believe that for a given lymphoid organ this proportion varies very greatly from rat to rat. In a system in which the number of cells susceptible to complement remained relatively constant, differences between curves of the type shown in Fig. 4 would reflect differences between different sera rather than

differences in the number of cells being attacked. However, if the complement content of serum were constant the curves could be used to provide an index of the number of cells being attacked (D. R. Forsdyke, unpublished work).

Time-course of inhibition

Fig. 5 shows the time-course of labelling with [^3H]uridine of cells grown at high concanavalin A concentrations in the presence or in the absence of complement. In the first hour of culture labelling was enhanced by concanavalin A to a similar extent in both the presence and the absence of complement. In the second hour of culture inhibition of labelling appeared in the concanavalin A-treated cultures which contained complement and shortly after 2h of

Fig. 4. *Effect of various proportions of heated and unheated calf serum on the response of rat lymphocytes to high concanavalin A concentrations*

Rat lymph-node cells were incubated (6 h, 37°C) in the presence (●) or in the absence (○) of concanavalin A (200 µg/ml of culture) with [³H]uridine (1 µCi/ml; 20 Ci/mmol) in various proportions of unheated and preheated (56°C, 30 min) calf serum, while the total serum concentration was kept constant (20%). Three different batches of calf serum were evaluated (Figs. 4a, 4b and 4c). In Figs. 4(a) and 4(b) cells from two rats were pooled and the final cell concentration was 2.3×10^6 /ml. Fig. 4(c) shows results from a separate experiment in which the final cell concentration was 1.6×10^6 /ml. Each point is the mean of triplicate cultures ($\pm$ S.E.M.). In the case of points presented without the S.E.M., the limits fell within or close to the area covered by the point.

Fig. 5. *Effect of high concanavalin A concentrations on the time-course of labelling with [³H]uridine of rat lymphocytes cultured in the presence of complement*

Rat lymph-node cells (1×10^6 /ml) were incubated for various periods in calf serum (20%) with [³H]uridine (1 µCi/ml) in the presence (●, ▲, ■) or in the absence (○, △, □) of concanavalin A (200 µg/ml of culture). The calf serum was preincubated (37°C, 2 h) either alone (■, □), or with cobra-venom complement inhibitor (12 µg/ml; ▲, △), or was preheated at 56°C for 30 min (●, ○). The mean radioactivity values (c.p.m.) of triplicate cultures are presented directly without the S.E.M.

culture the labelling was depressed below that of control cultures. Thus an initial complement-independent phase of lymphocyte activation by concanavalin A precedes a complement-dependent phase of lymphocyte inhibition by concanavalin A.

The inhibition was further examined by prelabelling cells with [³H]uridine, washing the cells free of unincorporated [³H]uridine, and then observing the effects of concanavalin A in the presence or in the absence of complement on the retention of acid-precipitable label by cells (Fig. 6a). Cells grown without concanavalin A showed a slow complement-independent loss of label over the 6 h incubation period. Cells grown with concanavalin A in preheated serum showed a similar rate of loss of label for the first hour of culture followed by a slightly decreased rate of loss of label. Cells grown with con-

Fig. 6. Effects of high concanavalin A concentrations in the presence or in the absence of complement on (a) the time-course of release of radioactive label from cells prelabelled with [^3H]uridine, and (b) the corresponding time-course of the appearance of label in the culture medium

Rat lymph-node cells ($15 \times 10^6/\text{ml}$) were prelabelled by incubating with [^3H]uridine ($4 \mu\text{Ci}/\text{ml}$; $30 \text{ Ci}/\text{mmol}$) for 2 h in preheated calf serum (10%). The cells were then pelleted by centrifugation (300g, 4 min), washed once by resuspension in warm medium 199 (37°C), and finally resuspended in medium 199 for immediate addition to master culture vessels containing either preheated or unheated calf serum (20%). The final cell concentration was $1.7 \times 10^6/\text{ml}$. After the addition of concanavalin A ($200 \mu\text{g}/\text{ml}$ of culture), 1 ml samples were pipetted into culture tubes which were incubated at 37°C. At various time-points cultures were harvested for the determination of (a) acid-precipitable radioactivity remaining in the cell pellets, and (b) the radioactivity in the culture medium. Each point is the mean of triplicate cultures ($\pm \text{S.E.M.}$). $\circ$, Preheated serum without concanavalin A; $\bullet$, preheated serum with concanavalin A; $\square$, unheated serum without concanavalin A; $\blacksquare$, unheated serum with concanavalin A.

canavalin A in unheated serum showed a similar rate of loss of label for the first hour of culture and then an accelerated loss of label occurred; by the sixth hour of incubation most of the original label had been lost. The lost label was quantitatively recovered from the culture medium (Fig. 6b), indicating that RNA breakdown products had not been retained within cells. Control experiments showed that the preincubation and washing procedures did not interfere with the ability of cells to respond to concanavalin A in heated serum.

Mechanism of attack on cells by concanavalin A and complement

These results are compatible with a mechanism by which concanavalin A molecules first bind to cells

and activate them. Subsequently, the binding of sufficient concanavalin A molecules would create conditions such that early components of the complement sequence could begin to bind to cells. When the terminal components of the sequence had bound, cell permeability would increase, ribonucleases would be activated and the breakdown products of RNA would appear in the medium (Fig. 6b). Early changes in the RNA content of nucleated cells attacked by complement have been reported by others (Green *et al.*, 1959a,b; Güttler & Clausen, 1969). Since our studies were performed with a culture medium with a high protein content, it is unlikely that intact RNA molecules were released from cells (Green *et al.*, 1959a,b). Although activation of endonucleases capable of initiating strand cleavage of DNA may occur quite early after complement attacks

nucleated mammalian cells (Shipley *et al.*, 1971), degradation of DNA to a non-acid-precipitable form occurs much later (Green *et al.*, 1959*a,b*). In keeping with this, no loss of DNA was detected in cultures during the first 6 h of culture (Milthorp & Forsdyke, 1970*b*).

The details of the conditions required for high, but not low, concanavalin A concentrations to cause complement to bind to cells remain to be determined. Possible conditions include the following. (i) Concanavalin A may be acting as an antigen analogue reacting in an immunologically non-specific manner with the antigen-specific immunoglobulin receptors produced and borne by lymphocytes with the potential to respond immunologically (Milthorp & Forsdyke, 1970*b*). Conformational changes in these receptors would permit complement to bind to the cell. In this respect it should be noted that concanavalin A reacts particularly well with immunoglobulin M (Harris & Robson, 1963; Morse, 1968), and that immunoglobulin molecules with characteristics of immunoglobulin M are likely candidates for the role of specific cell-borne antigen receptors (Vitetta *et al.*, 1971). Further, it has been shown that complement is required for inhibition of lymphocytes by high concentrations of specific antigen (Forsdyke, 1971*b*, and unpublished work). (ii) Either before or after it has bound to the cell, concanavalin A may react with factors in serum (such as natural anti-concanavalin A antibody), which in turn could mediate the binding of complement to cells. (iii) Concanavalin A may facilitate the binding of complement by natural anti-(rat lymphocyte) antibodies present in the calf serum. The failure to obtain the inhibition by high concanavalin A concentrations in homologous serum (Fig. 3*a*) could be construed as supporting the latter hypothesis. However, preliminary studies indicate that calf lymphocytes are inhibited by concanavalin A in autologous serum which suggests that rat serum may contain negative factors preventing the inhibition.

Effect of preheating calf serum on concanavalin A dose-response curves at different times of culture

Fig. 7 shows concanavalin A dose-response curves at different times of culture with either preheated or unheated calf serum. In the early hours of culture the response was assessed by using [³H]uridine; at later times 6–20 h 'pulses' of [³H]thymidine were employed. In unheated calf serum the optimum response occurred at the same concanavalin A concentration at all times of culture. In preheated serum there was no inhibition at high concanavalin A concentrations during the early hours of culture (Fig. 7*a*); subsequently an inhibition appeared so that the concanavalin A concentration at which a maximum response occurred progressively shifted to lower concanavalin

Fig. 7. Effect of preheating calf serum on concanavalin A dose-response curves at different times of culture

Rat lymph-node cells (1.2×10^6 /ml) were incubated for various periods at 37°C with various concentrations of concanavalin A in medium containing either unheated (○) or preheated (56°C, 30 min; ●) calf serum (20%). The response was assessed during the first 6 h of culture (Fig. 7*a*) by using [³H]uridine (1 μCi/ml; 24 Ci/mmol) present since the initiation of culture. At subsequent time-points the response was assessed with [³H]thymidine (1 μCi/ml; 23.3 Ci/mmol) added 6–20 h before the cultures were harvested. The labelling periods with [³H]thymidine were 24–30 h (Fig. 7*b*), 48–68 h (Fig. 7*c*) and 68–76 h (Fig. 7*d*). Each point is the mean of duplicate cultures.

A concentrations (Figs. 7*b* and 7*c*). Eventually the concanavalin A concentration required for an optimum response became the same as that in unheated serum (Fig. 7*d*); however, the magnitude of the response was still greater in preheated serum.

Thus at high concanavalin A to serum ratios cells

can be inhibited in preheated serum, but for this inhibition to appear prolonged incubation is required. Possible explanations for the inhibition include the following. (i) Given sufficient time high concanavalin A concentrations could exert a direct inhibitory effect on cells independent of complement (e.g. Fig. 2). (ii) During prolonged incubation cells might secrete complement components (Wyatt *et al.*, 1972) in sufficient quantities to overcome any complement inhibitory activity in heated serum. This complement would mediate an inhibition similar to that seen in the early stages of culture in unheated serum. (iii) The culture medium of cells growing in heated serum at optimum concanavalin A concentrations might become depleted of nutrients necessary for cell survival before the culture medium of cultures containing other than optimum concentrations of concanavalin A becomes depleted. The latter explanation was eliminated by the experiments described below.

Exhaustion of culture medium: the 'ceiling effect'

In studies with phytohaemagglutinin by using an assay of the total RNA content of cultures (Forsdyke, 1967, 1968), it was found that during the second day of culture dose-response curves showed a peak at the optimum phytohaemagglutinin concentration; subsequently dose-response curves showed a central plateau and eventually two peaks appeared on either side of the position of the original central peak. This two-peak phenomenon was also noted by several other workers (e.g. Havemann *et al.*, 1970b) and a variety of explanations were offered. The following interpretation of the results (Forsdyke, 1967) has gained independent support (Nordman, 1971).

In cultures which are not replenished with fresh medium there is a 'ceiling' on continued growth caused either by the exhaustion of medium nutrients or by the accumulation of some inhibitory metabolite. Most actively growing cultures containing optimum phytohaemagglutinin concentrations reach this ceiling first and further growth in such cultures is prevented. Cultures containing phytohaemagglutinin concentrations above and below the optimum continue growth until they too reach the ceiling. Dose-response curves at this stage of culture show a central plateau rather than a peak. The arrested growth is followed by the degeneration of cultures and this is initiated in cultures which reach the ceiling first. Thus there is a central depression in the plateau so that dose-response curves late in the culture period show two peaks. If studies had been carried out over only a low range of phytohaemagglutinin concentrations, dose-response curves would simply show a shift of the optimum response to lower phytohaemagglutinin concentrations, similar to that observed with concanavalin A in heated serum (Fig. 7). It is probable that the ceiling is due to the

Fig. 8. Effect of cell concentration on concanavalin A dose-response curves obtained with heated calf serum on the third day of culture

Rat lymph-node cells were incubated at cell concentrations of either 1.3×10^6 /ml ($\circ$), or 2.6×10^6 /ml (Δ), or 3.9×10^6 /ml ($\blacksquare$), in preheated (56°C , 30 min) calf serum (20%). [^3H]Thymidine ($1 \mu\text{Ci/ml}$; 23.3 Ci/mmol) was added for the last 12 h of the 72 h culture period. Each point is the mean of duplicate cultures.

exhaustion of medium nutrients, since the time of reaching the ceiling coincides with the time of exhaustion of the uridine competitor pool in the system (Forsdyke, 1968). The period required to reach the ceiling may vary with the species studied and can be increased by increasing the cell concentration, thus accelerating the rate of depletion of nutrients (Forsdyke, 1967; Nordman, 1971).

To study this in the present system, concanavalin A dose-response relationships were examined by using preheated calf serum and different cell concentrations. [^3H]Thymidine was added for the last 12 h of the 70 h culture period. At cell concentrations up to 2.6×10^6 /ml dose-response curves showed a clear central peak and doubling the cell concentration doubled the degree of radioactive labelling at all concanavalin A concentrations employed (Fig. 8). This direct linear relationship between cell concentration and labelling was also observed at the highest cell concentration examined (3.9×10^6 /ml), but only at sub-optimum and super-optimum concanavalin A

concentrations; the labelling in cultures containing around optimum concentrations of concanavalin A did not exceed that obtained with 2.6×10^6 cells/ml. At the optimum for lower cell concentrations the labelling with 3.9×10^6 cells/ml was depressed so that two peaks were evident.

Thus the ceiling effect is demonstrable in cultures with concanavalin A, but the cell concentration and culture period required to produce the phenomenon are in excess of those required to demonstrate the shift of the optimum response in heated serum (Fig. 7). The latter inhibition becomes progressively greater between 24 and 72 h of culture. This would be expected if a factor limiting the inhibition, such as complement, were secreted by cells. On the other hand, the exhaustion of medium nutrients would be expected to occur at a discrete moment in time (Forsdyke, 1968).

Biological significance

Theories of cell population homeostasis generally require effector elements by which the size of a cell population can be increased under one set of conditions and decreased under another set of conditions. We have shown here that concanavalin A can at low concentrations stimulate cell proliferation and at higher concentrations destroy cells, doing so by harnessing a natural agent lethal to cells, complement. We believe that our system provides a useful model for studying some aspects of the problem of cell-population homeostasis *in vivo*.

We thank Mrs. D. Lis and Mrs. Z. Vasa for technical help at various stages of this work, which was supported by grants from the National Cancer Institute of Canada and the Medical Research Council of Canada.

References

- Agrawal, B. B. L. & Goldstein, I. J. (1967) *Biochim. Biophys. Acta* **133**, 376–379
- Allan, D., Auger, J. & Crumpton, M. J. (1972) *Nature (London) New Biol.* **236**, 23–25
- Beckman, L. (1962) *Nature (London)* **195**, 582–583
- Forsdyke, D. R. (1966) in *Symposium on the Biological Effects of Phytohaemagglutinin* (Elves, M. W., ed.), pp. 115–123, R. Jones and A. Hunt Orthopaedic Hospital, Oswestry
- Forsdyke, D. R. (1967) *Biochem. J.* **105**, 679–684
- Forsdyke, D. R. (1968) *Biochem. J.* **107**, 197–205
- Forsdyke, D. R. (1971a) *Biochem. J.* **125**, 721–732
- Forsdyke, D. R. (1971b) *Proc. Can. Fed. Biol. Soc.* **14**, 35
- Götze, O. & Müller-Eberhard, H. J. (1971) *J. Exp. Med.* **134**, 90s–108s
- Green, H., Fleischer, R. A., Barrow, P. & Goldberg, B. (1959a) *J. Exp. Med.* **109**, 511–521
- Green, H., Barrow, P. & Goldberg, B. (1959b) *J. Exp. Med.* **110**, 699–713
- Güttler, F. & Clausen, J. (1969) *Biochem. J.* **115**, 959–968
- Harris, H. & Robson, E. B. (1963) *Vox Sang.* **8**, 348–355
- Havemann, K., Dosch, H. M. & Bürger, S. (1970a) *Z. Gesamte Exp. Med.* **153**, 297–307
- Havemann, K., Bürger, S. & Dosche, H. M. (1970b) *Z. Gesamte Exp. Med.* **153**, 308–323
- Marshall, W. R. & Roberts, K. B. (1965) *Quart. J. Exp. Physiol.* **50**, 361–374
- Milthorp, P. & Forsdyke, D. R. (1970a) *Nature (London)* **227**, 1351–1352
- Milthorp, P. & Forsdyke, D. R. (1970b) *Proc. Leukocyte Culture Conf. 5th* (Harris, E., ed.), pp. 143–171, Academic Press, London and New York
- Milthorp, P. & Forsdyke, D. R. (1972) *Proc. Can. Fed. Biol. Soc.* **15**, 147
- Morse, J. H. (1968) *Immunology* **14**, 713–724
- Nordman, C. T. (1971) *Scand. J. Clin. Lab. Invest.* **27**, Suppl. **116**, 49
- Nowell, P. C. (1960) *Cancer Res.* **20**, 462–466
- Rapp, H. J. & Borsos, T. (1970) *Molecular Basis of Complement Action*, 1st edn., Appleton-Century-Crofts, New York
- Shipley, W. U., Baker, A. R. & Colten, H. R. (1971) *J. Immunol.* **106**, 576–579
- Vitetta, E. S., Baur, S. & Uhr, J. W. (1971) *J. Exp. Med.* **134**, 242–264
- Walker, S. & Lucas, Z. (1971) *Proc. Leukocyte Culture Conf. 4th* (McIntyre, O. R., ed.), pp. 49–58, Appleton-Century-Crofts, New York
- Wyatt, H. V., Colten, H. R. & Borsos, T. (1972) *J. Immunol.* **108**, 1609–1614