Clin. exp. Immunol. (1979) 35, 433–442.

Deficiency in kwashiorkor serum of factors required for optimal lymphocyte transformation *in vitro*

D. W. BEATTY & E. B. DOWDLE *Department of Clinical Science and Immunology University of Cape Town, South Africa*

(*Received* 14 *August* 1978)

SUMMARY

Blastogenic responses of normal human peripheral blood lymphocytes cultured in media supplemented with serum from children with kwashiorkor were, on average, 47·7% of those observed when the same cells were cultured in the presence of normal AB serum. Incorporation of radioactive uridine was also diminished in the presence of kwashiorkor serum, indicating that lectin-induced RNA synthesis was also affected.

The kwashiorkor serum effect was not due to a cytotoxic action nor could it be attributed to the presence of saccharides or other inhibitors of the inducing lectins.

Mixing experiments showed that kwashiorkor serum was not inhibitory, but that it lacked factors present in normal serum that are required for optimal lymphocyte blastogenesis. The deficiency of these factors could largely be rectified by supplementing kwashiorkor serum with an ultrafiltrate of normal serum containing components with molecular weights of less than 500 Daltons.

We conclude that nutritional deprivation of severity sufficient to cause kwashiorkor leads to a deficiency of low molecular weight lymphocyte growth factors. This lack may contribute to the immunodeficiency associated with the disease.

INTRODUCTION

In a previous paper (Beatty & Dowdle, 1978) we reported that serum from children with kwashiorkor was deficient in its ability to support lymphocyte transformation *in vitro*. On the basis of these observations we suggested that defective cellular immune responses in this disease may be symptomatic of a nutritionally determined lack of some humoral factor.

In this paper we present experimental results that document, in greater detail, those respects in which kwashiorkor serum fails to provide an adequate medium supplement for *in vitro* lymphocyte transformation. Our data indicate that kwashiorkor serum is primarily deficient in some low molecular weight component or components that are present in normal serum and are required for optimal *in vitro* blastogenesis.

PATIENTS AND METHODS

Patients. Serum samples were obtained from seventeen children with kwashiorkor whose ages ranged from 12 to 50 months. Their expected weight for age ranged from 46–79%; serum albumin levels ranged from 1·5 g/100 ml to 2·2 g/100 ml. They were, in all essential clinical and biochemical respects, similar to the patients reported in our previous study (Beatty & Dowdle, 1978).

Normal serum was obtained from a pool of healthy, male, Rh +ve group AB blood donors.

Correspondence: Dr E.B. Dowdle, Department of Clinical Science and Immunology, University of Cape Town Medical School, Observatory 7925, Cape Town, South Africa.

0099-9104/79/0030-0433$02.00

Serum samples were heat inactivated at 56°C for 30 min, sterilized by filtration through a 0·45 μ Millipore filter and stored at −80°C until used.

Lymphocyte cultures. All cells used in this study were isolated by the method of Böyum (1974) from defibrinated venous blood obtained from healthy adults.

Triplicate lymphocyte cultures were established in stoppered 11 × 70 mm tubes (Nunc Roskilde, Denmark) or in round-bottomed microtitre plates (Dynatech, Zurich, Switzerland), depending upon the nature of each experiment. Tube cultures routinely contained 3×10^5 cells in 1 ml medium and were processed as previously described (Beatty & Dowdle, 1978).

Microcultures routinely contained 2×10^5 cells and 0·5 μg of PHA (Welcome purified, Beckenham, England) in a final volume of 0·2 ml in each microtitre well. Radioactive lymphocytes were harvested on glass fibre filter paper sheets (Skatron, Lierbyen, Norway) using a multiple automatic sample harvester after 72 hr in culture. Twenty-four hr before completing the incubation period 0·075 μCi ^{14}C thymidine (Radiochemical Centre, Amersham, England; specific activity 60 mCi/mmole) was added to each well.

In most cases, lymphocytes were cultured in Eagle's minimal essential medium (Gibco, New York.) buffered with 0·025 M Tris-HCl (T-MEM) and supplemented with penicillin (100 u/ml), streptomycin (100 μg/ml), glutamine (20 mM) and serum. Stoppered tubes or covered microtitre trays sealed with plastic wrap were incubated at 37°C in an atmosphere of air.

Deviation from these protocols and details of additions to the incubation media are given, for each experiment, in the results section.

Assay procedures. Measurement of ^{14}C or ^{3}H incorporated into harvested cells was performed by counting the dried glass fibre discs in 3 ml of scintillator solution (Instagel, Packard Instument Co., Downers Gove, Illinois) in a Tricarb scintillation spectrophotometer (Packard Instrument Co., Downers Grove, Illinois).

Cell viability was measured by trypan blue exclusion (Ling & Kay, 1975).

Nuclear size distribution and total viable cell counts in cell cultures were obtained by the method of Stewart, Cramer & Steward (1975), in which dead cells were digested with pronase. Viable cell nuclei were released by treatment with detergent and counted on an electronic particle counter (Coulter Electronics ZBI, Haileah, Florida) equipped with a multichannel pulse-height analyser for particle sizing. This method gave reliable and reproducible counts without the counting difficulties ordinarily associated with agglutination of cells in lectin-stimulated cultures.

Cells labelled with ^{3}H-thymidine were prepared for autoradiography by washing, suspending in AB serum and streaking on clean microscopic slides. The slides were coated with Kodak NBT2 liquid emulsion and exposed at −20°C for 7 days. Cells showing ten or more silver grains over the nuclei were scored as blasts; 200 cells were examined for each culture.

RESULTS

The effects of kwashiorkor serum on mitogen-induced lymphocyte transformation

The PHA-stimulated responses of normal adult lymphocytes cultured in sera from seventeen kwashiorkor patients and in normal AB serum are given in Table 1.

The average incorporation of radioactive thymidine in the presence of kwashiorkor serum was 47·7% of the value obtained in AB serum and ranged from 24–79%. These results are comparable to the values obtained in our preceding paper (Beatty & Dowdle, 1978) where the average value obtained in kwashiorkor serum cultures was 42·9% of that in AB serum cultures.

The effects of kwashiorkor serum on lymphocyte transformation were not affected by storage at −80°C and could be demonstrated in RPMI-1640 or Eagle's medium buffered with Tris-HCl or HEPES.

Effects of mitogen and mitogen concentration

Since mitogen-induced lymphocyte transformation is quantitatively related to the concentration of the mitogenic agent, inferior blastogenic responses in the presence of kwashiorkor serum may have been due to an oligosaccharide or other competitive inhibitor that effectively reduced the concentration of stimulating mitogen. To explore this possibility cells were stimulated with varied concentrations of three mitogens with different specificities (PHA, Concanavalin A and pokeweed mitogen) in the presence of AB serum and kwashiorkor serum.

As can be seen from the results summarized in Fig. 1, lymphocyte blastogenesis was lower in kwashiorkor serum irrespective of the mitogen used. Furthermore, concentration/response curves in kwashiorkor serum were not displaced to the right as would have been expected had there been an inhibitor of mitogen action present.

TABLE 1. PHA-induced transformation of normal lymphocytes in the presence of normal AB serum and seventeen samples of kwashiorkor serum. In each experiment 2×10^5 cells were incubated in microtitre plates in a final volume of 200 μl of T-MEM supplemented with 12·5% AB or kwashiorkor serum. Cells from different normal donors were used for each experiment. A single pool of AB serum was used throughout. Each 'kwashiorkor serum' value was obtained with a serum sample from a different patient. Results are the mean values of triplicate cultures in d/min.

Expt.	AB serum	Kwash. serum	Percentage of Kwash./AB
1	14934	5997	40·2
2a	34903	9428	27·0
2b	34903	8479	24·3
3a	10300	5769	56·0
3b	10300	5784	56·2
4	25763	20524	79·7
5	9122	5240	57·4
6a	11660	4923	42·2
6b	11660	6871	58·9
7	31271	16210	51·8
8	14832	3897	26·3
9	5536	2222	40·1
10	14881	6389	42·9
11	8807	5858	66·5
12	27556	17714	63·6
13	18917	7142	37·8
14	6043	2429	40·2
Average	17158·1	7933·9	47·7

The effect of kwashiorkor serum on the kinetics of RNA and DNA synthesis

In order to determine whether depressed lymphocyte responses in kwashiorkor serum were due to a delay in the initiation of RNA or DNA synthesis by PHA-stimulated lymphocytes, pulse experiments were performed in which ^{3}H-uridine (0·5 μCi; 40 Ci/mmole) or ^{3}H-thymidine (2·0 μCi; 2 Ci/mmole) were added to cultures at various times and the cells harvested 2 hr after the addition of radioactive nucleic acid precursors. The results of these experiments, given in Fig. 2, showed that both DNA and RNA synthesis were depressed in the presence of kwashiorkor serum. There was, however, no delay in the initiation of synthesis of these macromolecules.

The effects of kwashiorkor serum on cell viability and proliferation

Trypan blue exclusion tests on cells cultured in normal and kwashiorkor serum showed no differences in percentage viability in the two populations examined daily during a 5-day culture period. In all cases the percentage viability was greater than 90%.

When the method of Stewart, Cramer & Steward (1975) was used to determine total viable cell counts, the results depicted in Fig. 3 were observed. After an initial drop in numbers of viable cells in both sets of cultures during the first 24 hr, cell numbers in cultures supplemented with AB serum increased progressively from day 1 to reach a density of $1·7 \times 10^6$ cells/ml on day 6. Cells cultured in the presence of kwashiorkor serum showed a modest proliferation between day 1 and day 3 after which cell numbers remained static at $0·8 \times 10^6$/ml.

Effect of kwashiorkor serum on blastogenesis as measured directly

Although incorporation of radioactive thymidine into a population of cultured cells provides an

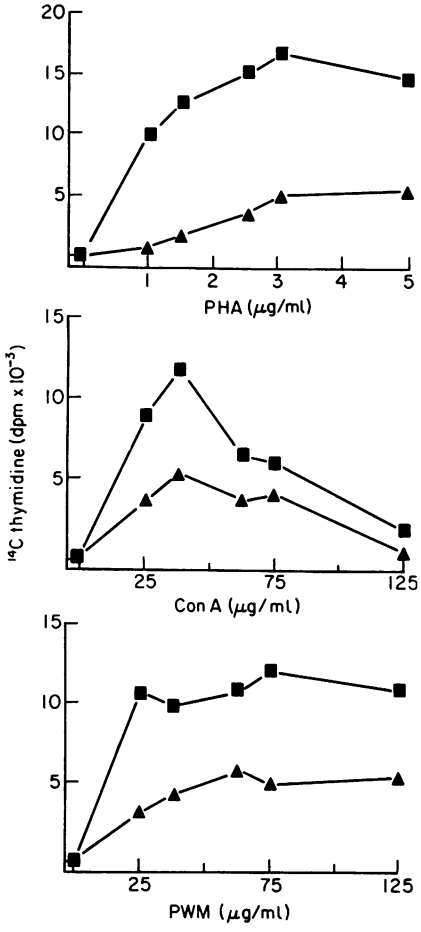

FIG. 1. Blastogenic responses of 2×10^5 normal human lymphocytes, cultured in medium supplemented with 12·5% AB serum (■—■) or 12·5% kwashiorkor serum (▲—▲), plotted as a function of mitogen concentration. Cells were incubated in the presence of the indicated concentration of PHA and Con A for 3 days and of PWM for 6 days.

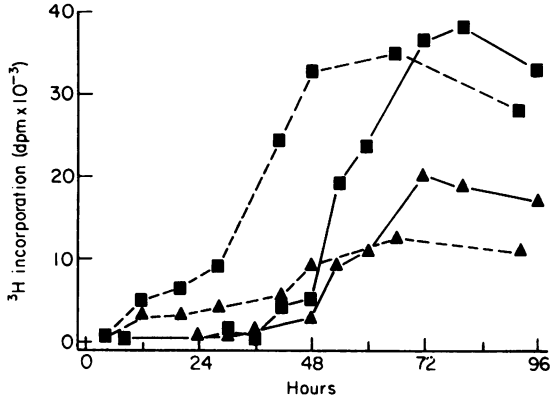

FIG. 2. Kinetics of PHA-induced incorporation of ^{3}H-uridine (- - - - - - - -) and ^{3}H-thymidine(———) into 2×10^5 normal peripheral blood lymphocytes cultured in the presence of 12·5% AB serum (■) or 12·5% kwashiorkor serum (▲). Radioactive nucleosides were adddd to micro cultures 2 hr before harvesting at the times shown.

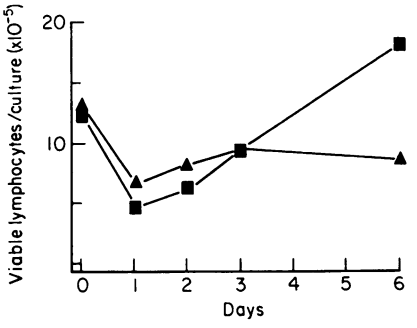

FIG. 3. Numbers of viable PHA-stimulated lymphocytes plotted as a function of time in culture in the presence of 12·5% AB serum (■—■) or 12·5% kwashiorkor serum (▲—▲).

objective and reliable means of quantifying mitogen-responsiveness, it is generally desirable to confirm such results with direct procedures that enumerate cells that have undergone blastic transformation. This was particularly so in the present context, since discrepant results have been obtained with radioactive measurements and blast-cell enumeration in studies of malnutritrition (Burgess *et al.*, 1974).

Two series of experiments were performed, therefore, to examine the effect of kwashiorkor serum on lymphocyte transformation as measured by an increase in nuclear size and by autoradiographic techniques.

In the first series, 3-day cultures were treated with pronase and cetrimide and the size distributions of the nuclei so obtained were determined with an electronic particle counter equipped with a multichannel analyser. As can be seen from a typical experiment summarized in Fig. 4, nuclei from unstimulated cells had a relatively narrow size-distribution restricted to channels corresponding to smaller particles. Nuclei from cells stimulated with PHA in AB serum showed a broad, bimodal size distribution with a distinct fall in numbers of smaller cells and a prominent increase in the numbers of larger cells. Nuclei from cells stimulated with PHA in the presence of kwashiorkor serum showed a size-distribution intermediate between that of unstimulated cells and those treated with PHA in AB serum.

In the second series, PHA-stimulated cells were exposed to 1 μCi/ml of ^{3}H-thymidine for the final 24 hr of a 3-day culture and autoradiographs of the cells were examined microscopically. Approximately 20% of the cells incubated in AB serum showed nuclear labelling (range 11·5%–23·5%), whereas less than 10% (range 0·6%–7·6%) of the cells incubated in kwashiorkor serum underwent blastogenesis by this criterion (Fig. 5).

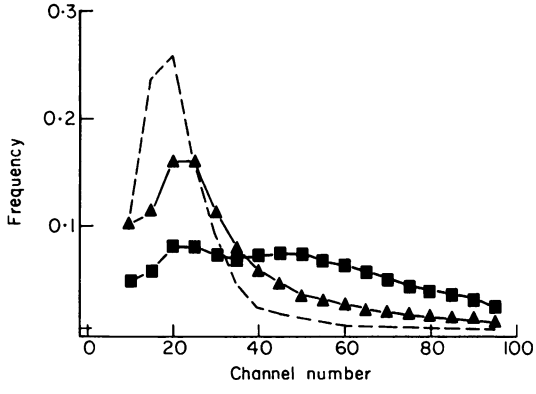

FIG. 4. Size distributions of lymphocyte nuclei after culture in media containing either 20% AB serum without PHA (- - - - -); 20% AB serum with 1·0 μg/ml PHA (■—■); or 20% kwashiorkor serum with 1·0 μg/ml PHA (▲—▲). After 72 hr incubation, the cells were treated with pronase and cetrimide and the size distributions of the released nuclei determined with an electronic particle counter equipped with a multichannel analyser. Particle volume is directly related to channel number.

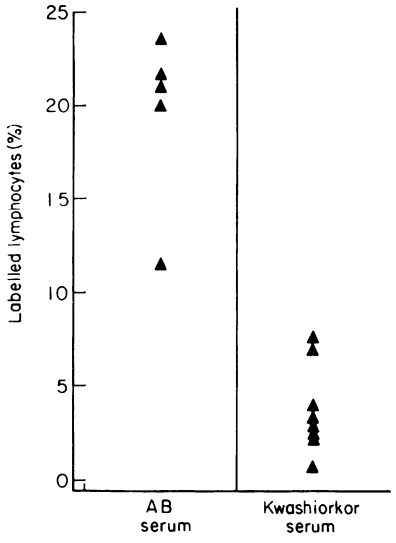

FIG. 5. PHA-induced lymphocyte blastogenesis in media supplemented with 20% AB serum or 20% kwashiorkor serum. Each point represents the results of autoradiographic evaluation of 200 cells from ^{3}H thymidine pulsed cultures of lymphocytes obtained from different normal donors. A pool of normal AB serum and eight different kwashiorkor sera were used to supplement culture media.

Serum switch during the mitogenic response

When the rate of radioactive thymidine incorporation into lymphocyte DNA is measured as a function of time after the addition of mitogen, one finds (Fig. 2) that there is a lag period of approximately 48 hr during which little or no DNA synthesis occurs, followed by a period of rapid incorporation lasting approximately 24 hr. Although the cellular and biochemical events involved in PHA-induced transformation are not known in any detail, one may infer, from the kinetic experiments, that the mitogenic response involves at least two phases—an early phase during which the responding cells interact with the lectin and proceed through a sequence of more or less synchronous events leading to a later phase of DNA assembly.

The experiment summarized in Fig. 6 was performed to see if cells in the early and late phases of the blastogenic response were equally susceptible to the effects of kwashiorkor serum. In this experiment, 2 ml of warmed serum-free medium was added to cultures after 24 hr or 48 hr of incubation. The cells were pelleted by gentle centrifugation; the medium was removed by careful aspiration; and the cells were resuspended in 1 ml of fresh medium supplemented with 'switched' serum—i.e. cells that had been incubated initially in kwashiorkor serum were switched to AB serum and vice versa. The cultures were then returned to the incubator for the remainder of the 72-hr experimental period. Control cells were either left undisturbed in the same serum throughout or washed without switching the medium. In all cases ^{14}C-thymidine was present during the last 24-hr period.

Cells incubated in AB serum throughout incorporated approximately 20,000 d/min of ^{14}C-thymidine; those incubated in kwashiorkor serum incorporated approximately 9000 d/min. Washing and resuspending the cells after 24 or 48 hr in culture had no significant effect on these control values. When AB serum was present during the first 24-hr period, 12,610 d/min were incorporated; when present during the last 24-hr period, 11,400 d/min were incorporated. Better responses were seen when AB serum was present during the first 48 hr (17,300 d/min) or the last 48 hr (14,900 d/min), but neither of these equalled those seen when AB serum was present throughout.

We conclude, therefore, that the effects of kwashiorkor serum were apparent during both the early and the late phases of the mitogenic response. Marginally superior responses encountered when AB serum was present during the first 24 hr or 48 hr compared to the last 24 hr or 48 hr indicates that cells in the early phase were perhaps more susceptible to the kwashiorkor serum effect than were those in the last phase.

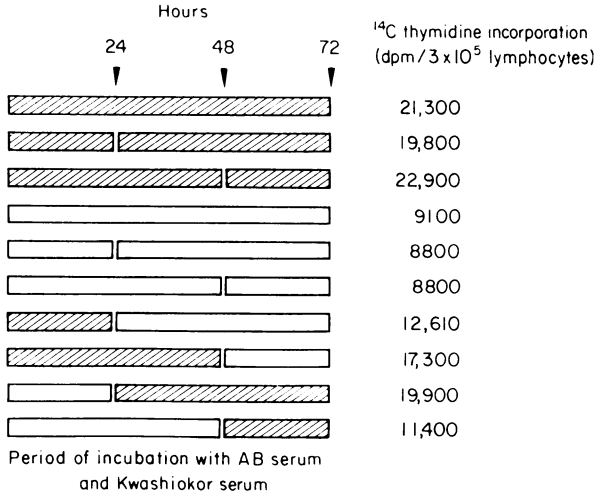

FIG. 6. Serum substitution experiments in which tube cultures of 3×10^5 lymphocytes were stimulated with 1·0 μg/ml of PHA and incubated for 72 hr in the presence of AB serum (▨) or kwashiorkor serum ( ) according to the protocols summarized diagrammatically in the figure. Vertical interruptions in the horizontal bars at 24 or 48 hr indicate times at which cells were pelleted and resuspended in fresh medium. Radioactive thymidine was added to cultures for the last 24 hr of incubation.

The effects of varying concentrations of AB serum, kwashiorkor serum or mixtures of the two on lymphocyte transformation

The inability of kwashiorkor serum to serve as an adequate medium supplement for optimal blastogenic responses may have been due to the presence of a serum inhibitor or to a deficiency of some essential component present in normal serum. To distinguish between these possibilities, mixing experiments were performed in which increasing acounts of kwashiorkor serum were added to fixed, limiting concentrations of normal serum.

By choosing concentrations of AB serum that would give thymidine incorporation results falling on the steep portions of the serum/response curve, conditions for the detection of inhibitors in the kwashiorkor serum were optimized.

Results of three such experiments are depicted in Fig. 7. When lymphocyte responses to PHA were measured with different concentrations of AB serum in the medium, an approximately sigmoid relationship was observed with a steep slope between 5% and 15% serum and a gradual slope between 15% and 25% serum. Kwashiorkor serum showed a similar relationship but in all cases the lymphocyte responses at any given serum concentration were lower than those observed with AB serum. When increasing amounts of kwashiorkor serum were added to tubes containing 5% or 7·5% AB serum, lymphocyte responses increased with increasing total serum concentration, albeit at a lower rate than in the tubes when AB serum was added. In no case was a significant *fall* in thymidine incorporation observed with increasing amounts of kwashiorkor serum.

These results indicate that the kwashiorkor sera we have tested were deficient in some component/s present in normal serum and required for optimal lymphocyte transformation. They have not been shown to contain inhibitors of blastogenesis.

To obtain an approximate estimate of the molecular size of the component/s in which kwashiorkor serum was deficient, lymphocyte responses were measured in media containing 5% kwashiorkor serum complemented with further additions of an ultrafiltrate of AB serum prepared with an Amicon UMO5 membrane (Amicon Corporation, Lexington, Massachusetts, m.w. cut-off <500 Daltons).

As can be seen from the results summarized in Fig. 8, addition of increasing amounts of AB ultrafiltrate to cultures containing 5% kwashiorkor serum progressively augmented lymphocyte transformation responses. Cultures containing 5% kwashiorkor serum and ultrafiltrate equivalent to 25% AB serum

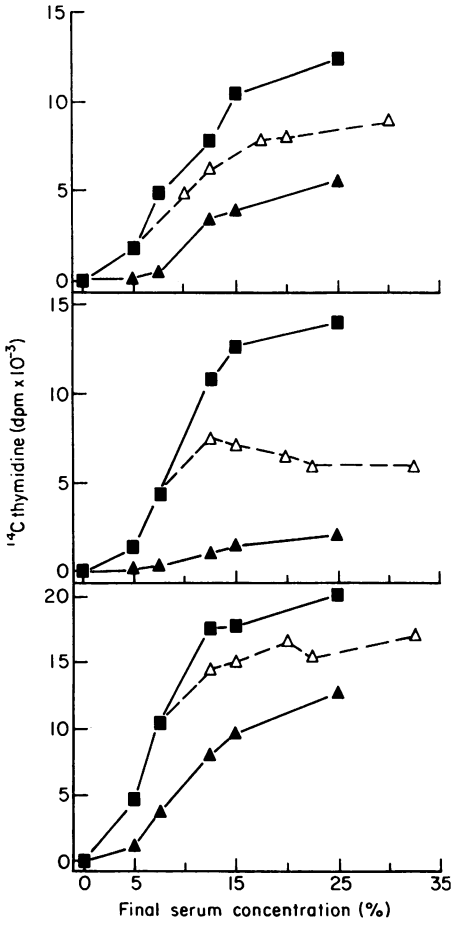

FIG. 7. PHA-induced ^{14}C-thymidine incorporation into 2×10^5 lymphocytes incubated in media supplemented with different concentrations of AB serum (■—■); with different concentrations of kwashiokor serum (▲—▲); or with a constant, limiting concentration of AB serum to which increasing amounts of kwashiokor serum were added (△ - - - △).

showed thymidine incorporation values that were higher than those observed with optimal concentrations of AB serum.

Since kwashiorkor serum is invariably deficient in albumin, an experiment was performed in which purified human serum albumin (4 g/100 ml) and an Amicon UMO5 serum ultrafiltrate were added, singly or together, to 5% kwashiorkor serum. Fig. 8 shows that neither human serum albumin nor ultrafiltrate, on their own or combined, were able to support lymphocyte transformation in serum-free medium. The addition of albumin alone to 5% kwashiorkor serum did not enhance lymphocyte transformation responses, and the combination of albumin and ultrafiltrate did not significantly increase the values obtained with ultrafiltrate alone.

These experiments suggest that the reduced lymphocyte transformation responses seen in cultures with kwashiorkor serum were mainly due to a deficiency of a low molecular weight serum fraction that was present in normal serum.

DISCUSSION

The experimental results we report in this paper confirm and extend our previous observations (Beatty & Dowdle, 1978). Serum samples from children with kwashiorkor were consistently deficient in their ability

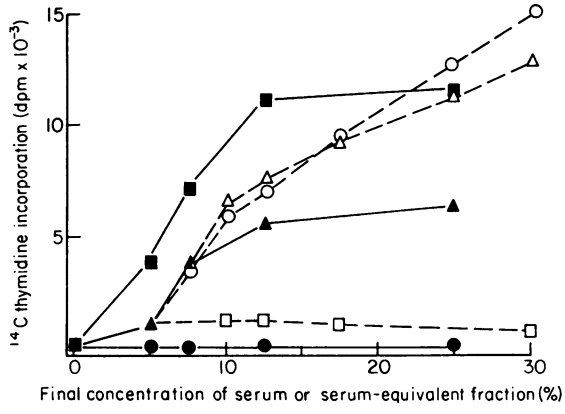

FIG. 8. PHA-induced ^{14}C-thymidine incorporation into lymphocytes incubated in media supplemented with sera or serum fractions alone or in combination. For comparative purposes albumin concentrations are depicted in terms of 'serum equivalents', 40 mg/ml of albumin being regarded as equivalent to 100% normal serum with respect to albumin concentration. Symbols as follows: AB serum alone (■—■); kwashiokor serum alone (▲—▲); 5% kwashiokor serum + addition of UMO5 ultrafiltrate of AB serum (△- - -△); 5% kwashiorkor serum + addition of UMO5 ultrafiltrate and albumin (○- - -○); 5% kwashiorkor serum + addition of albumin (□- · -□); albumin alone, ultrafiltrate alone or albumin + UMO5 ultrafiltrate (●—●).

to support lymphocyte transformation *in vitro* when compared to a reference pool of normal adult AB serum.

The kwashiorkor serum effect was not mitogen specific inasmuch as it affected blastogenic responses to PHA, PWM, Con A (Fig. 1) and allogeneic cells (Beatty & Dowdle, 1978). Lectin dose-response experiments showed that the serum did not act by inactivating or otherwise reducing the effective concentration of the mitogen (Fig. 1).

Kwashiorkor serum supplements gave inadequate blastogenic responses, not only as measured by ^{14}C-thymidine incorporation into whole cells, but also as determined by nuclear-sizing techniques and histological evaluation of autoradiographs. We conclude, therefore, that the serum effect was exerted directly upon nuclear DNA synthesis and not upon transport of thymidine into stimulated cells nor upon the size of intracellular thymidine pools.

Mixing experiments showed that the defect in kwashiorkor serum was due to a deficiency of some factor/s essential for optimal lymphocyte transformation and not due to the presence of an inhibitor. This observation is intuitively consistent with the concept of kwashiorkor as a deficiency disease in which nutritional deprivation hampers all growth and the cellular proliferation that this implies (Ramalingaswami, 1969). Reports of humoral factors affecting *in vitro* lymphocyte responses have recently been collated by Nelson & Gatti (1976) in an extensive review which discusses factors found in pathological human sera, normal human sera and animal sera that act as inhibitors of blastogenesis. The phenomenon we have documented with kwashiorkor serum does not come under consideration in this category.

The results of viable cell counts and the results of serum mixing experiments, enable us to exclude the trivial explanation that kwashiorkor serum was simply cytotoxic for lymphocytes. The precise manner in which the deficiency in kwashiorkor serum acted, however, is obscure. The serum switch experiments indicated that optimal transformation required the presence of normal serum throughout both initial and late phase of the mitogenic response, although there was some indication that the initial, pre-synthetic phase was preferentially affected by the deficiency in kwashiorkor serum. It is possible that better synchronized cultures might have identified, with more certainty, a specific period in the cell cycle during which stimulated cells were particularly vulnerable to the effects of the deficiency.

When PHA-stimulated lymphocytes were incubated in medium supplemented with kwashiorkor serum and harvested after 2-hr pulses of ^{3}H-thymidine or ^{3}H-uridine at different times after initiation of cultures, cellular incorporation of both radioactive nucleosides was depressed compared to cells incubated with normal serum (Fig. 2). These results indicate that the synthesis of both RNA and DNA were

affected by the inadequacy of the kwashiorkor serum. Since RNA synthesis precedes DNA synthesis in this system and is known to be required for S-phase DNA synthesis (Mueller, 1969), it is possible that the kwashiorkor serum effect on DNA synthesis was secondary to its effect upon prerequisite synthesis of RNA or other macromolecules.

We have, as yet, been unable to define the precise chemical nature of the compound/s that are lacking in kwashiorkor serum. These could, to a large extent, be supplied by the addition of an ultrafiltrate of AB serum, indicating that the kwashiorkor serum was mainly deficient in some low molecular weight (< 500 Daltons) component/s required for optimal transformation.

Since hypoalbuminaemia is a cardinal manifestation of kwashiorkor (Whitehead, Coward & Lunn, 1973) and since albumin has been used successfully as a serum substitute for lymphocyte transformation (Spieker-Polet & Polet, 1976) it was reasonable to suspect that a deficiency of this protein might have been partly responsible for the serum defect. This, however, did not prove to be the case, since the addition of albumin did not improve the supportive quality of kwashiorkor serum (Fig. 8).

Since the kwashiorkor serum deficiency was manifest in the presence of various tissue culture media, we conclude that the low molecular weight component/s that were lacking were not among those amino acids, vitamins, salts, energy substrates or other known growth factors normally included in the formulation of such media. The fact that tissue culture media lack low molecular weight materials that are necessary for optimum blastogenesis and are supplied by normal serum have been borne out by our observations (results not given) that AB serum that has been dialysed against RPMI 1640, Eagle's MEM or medium 199 loses its ability to support lymphocyte transformation. The experiments of Ling *et al.* (1965) gave results that allow the same conclusions.

Although serum factors that support lymphocyte transformation are complex and their positive and complete identification represents a formidable task, it is to be hoped that this final characterization will be achieved. The availability of such compounds in a pure, biologically active form would contribute much to the understanding and the management of the immunodeficiency of kwashiorkor.

We acknowledge with gratitude the excellent technical assistance provided by Mrs S. Gardner and the co-operation of the Western Province Blood Transfusion Service. Financial support for this study was obtained from the South African Medical Research Council.

REFERENCES

BEATTY, D.W. & DOWDLE, E.B. (1978) The effects of kwashiorkor serum on lymphocyte transformation *in vitro*. *Clin. exp. Immunol.* **32**, 134.

BÖYUM, A. (1974). Separation of blood leukocytes, granulocytes and lymphocytes. *Tissue Antigens*, **4**, 269.

BURGESS, B.J., VOS, G.H., COOVADIA, H.M., SMYTHE, P.M., PARENT, M.A. & LOENING, W.E.K. (1974) Radio-isotopic assessment of phytohaemagglutinin stimulated lymphocytes from patients with protein calorie malnutrition. *S. Afr. Med. J.* **48**, 1870

LING, N.R. & KAY, J.E. (1975) *Lymphocyte stimulation*. p. 36. *North Holland, Amsterdam.*

LING, N.R., SPICER, E., JAMES, K. & WILLIAMSON, N. (1965) The activation of human peripheral lymphocytes by products of staphylococci. *Brit. J. Haemat.* **11**, 421.

MUELLER, G.C. (1969) Biochemical events in the animal cell cycle. *Fed. Proc.* **28**, 1780.

NELSON, D.S. & GATTI, P.A. (1976) Humoral factors influencing lymphocyte transformation. *Progr. Allergy*, **21**, 261.

RAMALINGASWAMI, V. (1969) Interface of protein nutrition and medicine in the tropics. *Lancet*, **ii**, 733.

SPIEKER-POLET, H. & POLET, H. (1976) Identification of albumin as the serum factor essential for the growth of activated human lymphocytes. *J. Biol. Chem.* **251**, 987.

STEWART, C.C., CRAMER, S.F. & STEWARD, P.G. (1975) The response of human peripheral blood lymphocytes to phytohaemagglutinin. Determination of cell numbers. *Cell Immunol.* **16**, 237.

STEWART, C.C. & GOLDSTEIN, S. (1974) The direct determination of cytolytic antibody titre and specificity. *J. Lab. Clin. Med.* **84**, 425.

WHITEHEAD, R.G., COWARD, W.A. & LUNN, P.G. (1973) Serum albumen concentration and the onset of kwashiorkor. *Lancet*, **i**, 63.