the conceptus antigens may be a factor in the aetiology of pre-eclampsia. This theory does not seem to have been considered before and certainly was not in our minds at the outset. It may explain why the disease is more common in first pregnancies, which usually represent the first exposure to conceptus antigens.

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Details of the ABO and HLA typing of the patients in the study may be obtained from Professor J S Scott, Department of Obstetrics

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Immunological detection of residual leukaemic disease in the bone marrow of children with acute lymphoblastic leukaemia

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Summary and conclusions

Peripheral blood lymphocytes incubated with tumour cells or extracts may undergo blastogenesis. This is the basis of a technique studied in children with acute lymphoblastic leukaemia (ALL) in childhood in an attempt to predict relapse. Samples of peripheral blood and bone marrow from 82 children with varying degrees of ALL were analysed. Cultures were prepared by incubating a lymphocyte suspension with an autologous bone-marrow suspension. Final ratios of lymphocytes to bone-marrow cells (L : BM) were 1 : 1 and 2 : 1. Control wells received bone-marrow or lymphocyte suspension only. Cultures were incubated for 72, 96, and 120 hours. All were pulse-labelled with ³H-TdR and radioactivity was measured by scintillation counting. Results were expressed as the stimulation index, calculated by dividing the mean counts per minute (cpm) of wells containing both lymphocytes and bone-marrow cells by the sum of the mean cpm for control wells. If the stimulation index exceeded 1 at 72, 96, or 120 hours at either L : BM ratio a positive response was recorded.

Seventy-six children were in clinical remission at the time of testing (group A) and six were in clinical relapse (group B). In group A 24 patients showed stimulation

and relapsed later at a mean time of 3.8 months (21 with marrow disease, two with testicular infiltration, and one with lung infiltration). Sixteen patients showed stimulation and had up to 4% blasts in their bone marrow but remained in remission. Nineteen other patients showed a positive response and several factors may have contributed to this: two underwent a "rebound" lymphocytosis after stopping treatment, nine had current or intercurrent infections, two had persistent unexplained bone-marrow lymphocytosis, but six had no causative symptoms and thus their responses were "true false-positives." Seventeen patients from group A showed no response and remained in remission for a mean of 22.9 months after testing. None of the six children in group B responded, and at testing had 17-85% blasts in their bone marrow.

During the study no patient relapsed who had not shown a positive response. The technique merits further study as a guide to the presence of leukaemic cells.

Introduction

The reduction of lymphoblasts to 5% or less in bone-marrow smears is one of the commonly used criteria¹ of clinical remission in acute lymphoblastic leukaemia (ALL). Blasts are recognisable mainly morphologically and rarely cytochemically,² and the limitations of these methods are well known. A more sensitive diagnostic technique than the staining methods provide is needed to detect such a small number of leukaemic blasts. Such a test would improve the clinical management of patients, since leukaemic cells detected in a patient otherwise considered to be in remission would indicate the need for additional or intensive chemotherapy, or both, which should increase the number of long-term survivors. The test could also be used in deciding whether to stop or continue chemotherapy in a particular case.

Peripheral-blood lymphocytes incubated with intact tumour cells or tumour-cell extracts may undergo blastogenesis,³ which is detected morphologically or by the increase of DNA synthesis.⁴ Autologous non-neoplastic cells have little or no such stimulatory effect. The difference has been interpreted as a

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demonstration of cellular immunity wherein in-vivo sensitised lymphocytes are stimulated by the "recall" antigens present on the surface of tumour cells. Lymphocytes from patients in remission may be stimulated in vitro by the "relapse" leukaemic cells.⁵ Blastogenesis of autologous peripheral-blood lymphocytes could therefore probably be induced by a very small number of residual blasts in the marrow of a leukaemic patient even though the patient is in complete remission. We report the first study of this kind to be undertaken on children with ALL.

Patients and methods

Bone-marrow and peripheral-blood lymphocytes—All the children investigated had ALL and were being treated according to established protocols (mostly those being studied by the Medical Research Council working party on childhood leukaemia). Regular examinations of bone marrow are included in all these protocols, and samples from every child in remission are taken at 10-12-week intervals for routine haematological evaluation. Extra samples of bone marrow (1-2 ml) and peripheral blood (10-15 ml) were obtained from 82 children while bone marrow was being collected for morphological examination. Mononuclear cells, and blasts if present, were purified from heparinised fresh bone marrow on a Ficoll-Triosil gradient (450 g for 30 minutes). After being washed three times in medium 199, the cell suspension was irradiated (1100 rads). The cells were resuspended at a density of 1×10^6 or 0.5×10^6 cells/ml tissue culture medium 199. Peripheral blood samples were obtained by venepuncture, defibrinated, and spun to remove serum. An equivalent volume of medium 199 was added to replace serum. Lymphocytes were separated on a Ficoll-Triosil gradient, and after being washed three times with medium 199 were resuspended at a concentration of 1×10^6 cells/ml.

Lymphocyte stimulation—On microtest II plates (Falcon Plastics) 100 μ l of lymphocyte suspension (1×10^5 cells) was incubated with 100 μ l of autologous bone-marrow suspension containing 1×10^6 or 0.5×10^6 bone-marrow cells/ml. Thus the final ratio of lymphocytes to bone-marrow cells (L : BM) in the test wells was 1 : 1 or 2 : 1. The control wells received bone-marrow or lymphocyte suspension only; 100 μ l of medium 199 was added to control wells to make up the volume to 200 μ l. Finally, 50 μ l of heat-inactivated fetal calf serum was added to both test and control wells. Plates were incubated for 72, 96, and 120 hours at 37°C in a humidified atmosphere containing 5% CO₂ in air. Although samples from 82 children were examined, the volumes of the samples available allowed only 35 patients to be studied at both L : BM ratios and all three incubation times.

Harvesting and calculations—Three hours before termination all cultures were pulse-labelled with ³H-TdR (1 μ Ci/well, specific activity 23Ci/mmol). Samples were harvested with a multiple cell-culture harvester (Cryotech). Radioactivity was measured by scintillation counting and the results were expressed as the stimulation index, which was calculated by dividing the mean counts per minute (cpm) in wells containing a mixture of lymphocytes and bone-marrow cells by the sum of the means of the cpm in control wells containing lymphocytes and bone-marrow cells alone. If the stimulation index exceeded 1 on at least one occasion—that is, at 72, 96, or 120 hours—at either L : BM ratio a positive response was recorded.

Results

The ability of autologous peripheral-blood lymphocytes to respond to autologous bone-marrow cells was influenced by the L : BM ratio and period of incubation in the 35 patients who were studied at both ratios and all three incubation times (table I). The mean stimulation index for 72, 96, and 120 hours at an L : BM ratio of 1 : 1 was 2.74 compared with 2.33 at a ratio of 2 : 1 ($P < 0.01$). At both ratios extending the incubation period resulted in an increased rate of response and a proportional increase in the stimulation index. Six of the 35 patients showed no response at either ratio or any incubation time: 16 children responded at all ratios and incubation times; and 13 children responded at one or more of the ratios and incubation times.

Table II shows clinical details of the 82 children at the time of testing. Remission was defined as freedom from symptoms and signs of leukaemia with a cellular bone marrow showing haemopoiesis and containing 5% or fewer lymphoblasts and stem cells. Twenty-four children who were in clinical remission at testing (responding group A1) showed a positive response and relapsed later. The mean time between detection of stimulation and relapse was only 3.8 months

TABLE I—Stimulation of peripheral-blood lymphocytes with autologous bone-marrow cells at three incubation periods and two ratios of lymphocytes to bone-marrow cells (L : BM) in 35 children with acute lymphoblastic leukaemia

Period of incubation (h)	No of patients responding at L : BM ratio 1 : 1	Mean stimulation index	No of patients responding at L : BM ratio 2 : 1	Mean stimulation index
72	22/35	1.56	18/35	1.26
96	26/35	2.13	22/35	1.97
120	28/35	4.53	26/35	3.75
Mean		2.74		2.33

Source of variation	Sum of squares	Degrees of freedom	Mean squares	F	Significance
Ratio	9.06050	1	9.06050	9.82	P0.001
Period of incubation	284.51050	2	142.25520	154.11	P0.001

TABLE II—Clinical details of 76 patients in remission (group A) and six patients in relapse at time of testing (group B)

	No of patients
Group A (responding):	
(1) In remission at testing but relapsed later	24
(2) In apparent clinical remission but up to 4% blasts in bone marrow; continued in remission	16
(3) Possible false-positive responses	19
Group A (non-responding):	
(1) In remission at testing; continued in remission for 22.9 months	17
Group B (non-responding):	
(1) All patients in relapse at testing	6
Total	82

(table III). Twenty-one of these patients had leukaemic blasts in their bone marrow. Blasts were not detected in the bone marrow of the remaining three patients, though testicular biopsy in two and lung biopsy in the third showed leukaemic cells. These three patients were therefore also considered to be in relapse. The interval before relapse was not correlated with the extent of stimulation—that is, higher stimulatory indices were not necessarily followed by quicker relapses. Sixteen patients who responded (responding group A2; table II) had up to 4% blasts in their bone marrow, though they were apparently in clinical remission; they continued in remission.

TABLE III—Interval between positive response to test and clinical relapse in 24 children in clinical remission from acute lymphoblastic leukaemia at time of testing. Mean interval was 3.8 months

Case No	Interval (months)	Case No	Interval (months)	Case No	Interval (months)
1*	2	9*	0	17	7†
2*	7†	10*	3	18*	6†
3*	5†	11*	5†	19*	0
4*	3	12	2	20*	0
5*	2	13	0	21	5††
6*	1	14	3	22	9††
7*	7†	15	3	23	7†§
8*	3	16	8†	24*	0

*Patients who subsequently died.

†No sample tested in period between testing and clinical relapse.

‡Testicular biopsy showed leukaemic cells.

§Lung biopsy showed leukaemic cells.

Bone-marrow samples from a third group of 19 children (responding group A3—table II) who were in clinical remission stimulated autologous lymphocytes. In two of these patients the bone marrow showed signs of the "rebound" effect—that is, increase in blasts or lymphocytosis—that sometimes follows the end of chemotherapy; nine had either recurrent or concurrent infections at the time of testing; and two others had persistent lymphocytosis in the bone marrow, the cause of which was unknown. The results in these 13 children were therefore regarded as "false-positive" because factors other than malignant disease caused the positive response. No explanation could be found for the stimulation in the other six children. Four of these were undergoing chemotherapy and the other two were off treatment.

In contrast to the 24 children in responding group A1, 17 patients (non-responding group A1) showed a negative reaction and remained

in remission for a mean of 22.9 months after testing. These patients had been in remission for 17.9 months before testing.

Only six of the 82 children studied were in clinical relapse at the time of testing (group B). None of them responded. The proportion of blasts in these children ranged from 17% to 85%. The negative results may have been due to antigen excess or to impaired cellular responses. To find the minimum percentage of relapse blasts needed to stimulate autologous peripheral blood lymphocytes, blasts were separated from bone-marrow samples from two patients in relapse and stored in liquid nitrogen in a freezing medium containing 10% dimethyl sulphoxide. After remission had been induced with cytotoxic drugs (both patients had had at least 87% blasts in their bone marrow) lymphocytes were collected from the peripheral blood and cultured in the presence of varying concentrations of stored autologous relapse blasts. The minimum percentage of blasts needed to produce a blastogenic response (stimulation index >1) was found to be $0.7 \pm SE$ 0.3 in one case and 1.21 ± 0.4 in the other.

Discussion

These results show that peripheral-blood lymphocytes from patients with ALL in remission can be stimulated by autologous bone-marrow cells. The mechanism of the stimulatory response is uncertain, but it is probably directed against antigens associated with leukaemic blasts that may not be detectable on routine haematology. Increasing evidence suggests that membrane-bound antigenic determinants are present on leukaemic cells but not on normal cells.⁶⁻⁹ The blastogenic response in our patients in remission was either associated with a few blasts (up to 4%) in the bone marrow or was quickly followed by relapse (mean time 3.8 months).

The bone marrow of all the 24 children who relapsed during the study (responding group A1) produced appreciable stimulation of peripheral-blood lymphocytes. Thus during the past 24 months we have seen no child whose response could be classified as false-negative—that is, one who had not responded before relapsing. The difference between the disease-free period of the 24 responding patients (table II) and the 17 children whose lymphocytes failed to respond to stimulation (non-responding group A1) was highly significant ($P < 0.001$). Gutterman *et al*¹⁰ cultured autologous blood lymphocytes with bone-marrow cells from 25 adults with acute leukaemia in remission. Out of 17 patients whose peripheral-blood lymphocytes failed to react to autologous bone-marrow cells, 15 remained in complete remission (median time 10.5 months); but five of the remaining eight patients whose lymphocytes were stimulated by the bone-marrow cells subsequently relapsed (median time 6.5 months). The difference in relapse rates between these two groups was also significant.

The failure of peripheral-blood lymphocytes to be stimulated by bone-marrow cells in the group of 17 non-responding children is easily explained by the long and continuous absence of leukaemic cells in the marrow. Two reasons might explain the non-stimulation of lymphocytes from children who were in relapse when the in-vitro test was performed. Firstly, some blasts from the bone marrow may have escaped into the circulation causing an antigen excess or blocking effect so that the addition of blasts did not have any blastogenic influence. Secondly, the cellular responses of children with ALL are extremely low when in relapse but return to normal immediately after induction of remission.^{11 12} We have not tested the ability of lymphocytes from patients in relapse to respond to other antigens.

Bone-marrow samples from six of the 17 non-responding children, who continued in complete clinical remission for an

average of 23 months, were tested at regular intervals. All of the 26 repeat tests on these long-term survivors failed to induce blastogenesis of autologous peripheral-blood lymphocytes. This correlation between a lack of response and absence of leukaemic blasts suggests that a negative result in the in-vitro test is a good prognostic sign. Nevertheless, a blastogenic response among patients in remission does not necessarily indicate the presence of leukaemic blasts, nor is it a certain sign of ensuing relapse, since in our study 13 children who responded had no signs of malignant disease but had symptoms such as infections, lymphocytosis, and "rebound" effect. Other primary and secondary haematological disorders may also produce a positive response, but as such studies are limited for ethical reasons no healthy controls have yet been examined.

Interestingly, three asymptomatic patients with no excess of marrow blasts showed a positive response to testing. Biopsy specimens from other than the routine sites (namely, bone marrow and CSF) showed leukaemic infiltrates (cases 21-23—table III). In one patient a lung biopsy was performed for the diagnosis of nodular lung disease and histological examination showed leukaemic cells. Testicular biopsy specimens from two other boys also contained leukaemic cells. In another patient (table III—case 12), who had recurrent bone pain, the bone marrow showed relapse at one site, though other sites were normal. Marrow samples taken from two other sites over a prolonged period were normal.¹³ Bone-marrow cells from these three apparently normal aspirates produced stimulation of autologous peripheral-blood lymphocytes.

Patients in apparent clinical remission may have a few leukaemic cells in extramedullary sites.¹⁴ Simple bone-marrow aspiration may not provide enough evidence of a disease-free state and patients with a positive blastogenic response may still be harbouring leukaemic cells and hence be in danger of ultimate relapse. A positive response may be relevant to the issue of whether to stop or continue treatment or to add extra treatment such as testicular irradiation.

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