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Immunology

Demonstration of the inhibitory effect of human alpha-fetoprotein on *in vitro* transformation of human lymphocytes*

(phytomitogens/mixed lymphocyte culture/serum protein/fetal development)

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ABSTRACT **We have studied the effects of human alpha-fetoprotein (HAFP), isolated from the serum and ascitic fluid of a hepatoma-bearing patient, on the *in vitro* transformation of human peripheral blood lymphocytes by a variety of mitogenic stimuli. At a concentration of 2.5 mg/ml, HAFP inhibited the lymphocyte response to phytohemagglutinin, concanavalin A, and rabbit anti-human thymocyte serum, but failed to inhibit the response to pokeweed mitogen. HAFP was able to inhibit the one-way mixed lymphocyte culture at concentrations of 250–500 μg/ml, but failed to inhibit at 100 μg/ml. Exposure of lymphocytes to 2.2 mg/ml of HAFP for 18 hr did not result in significant lymphocytotoxicity, and such cells washed free of HAFP were fully capable of participating in the mixed lymphocyte culture. HAFP did not inhibit lymphocyte E-rosette formation. Fetal HAFP was more effective in inhibiting human lymphocyte responses than hepatoma HAFP. These experiments support the suggestion that HAFP plays an important immunoregulatory role during fetal development, possibly through the suppression of thymus-derived lymphocyte responses to antigenic stimuli; they also suggest that there are important differences in the biological properties of hepatoma and fetal HAFP.**

Until recently, little was known of the biological role of the alpha-fetoprotein (1), although it was regarded as an embryonic serum albumin (2, 3) and was shown to possess estrogen hormone-binding properties (4). Our report on the inhibition of human lymphocyte transformation *in vitro* by fetuin (5), a bovine alpha-fetoprotein, and the simultaneous observations by Tomasi and his coworkers that murine alpha-fetoprotein suppresses murine lymphocyte responses *in vitro* to both mitogenic and antigenic stimuli (6, 7), suggest an immunoregulatory role for alpha-fetoproteins during fetal development.

This paper presents experimental data that demonstrate the inhibitory effects of human alpha-fetoprotein (HAFP) on lymphocyte responses *in vitro* to a variety of mitogenic stimuli which include the one-way mixed lymphocytes culture. The results suggest that this effect may be due primarily to an inhibition of thymus-derived lymphocytic (T-lymphocyte) function.

MATERIALS AND METHODS

The techniques for isolating, culturing, and harvesting cultures of human lymphocytes were previously described (5, 8–10). A 1 ml culture system was employed throughout, and all experiments with cultures were performed in triplicate.

HAFP was isolated from the serum and ascitic fluid of a patient with hepatoma, or from pooled fetal serum, by a modification of the method of Hirai *et al.* (11) using immunoadsorbent affinity chromatography.† Crystalline human serum albumin (Miles Laboratory, Kankakee, Ill.) was added to lymphocyte cultures as a control for nonspecific human serum protein (12), so that its effects could be compared with those of HAFP. Unless otherwise noted, all experiments were done with HAFP from hepatoma patients. Additions of HAFP or human serum albumin to lymphocyte cultures were made at the time of initiation of the cultures, and the cultures were gently mixed prior to the addition of the mitogenic stimulus.

The effect of preexposure with HAFP on the viability of human lymphocytes was determined by trypan blue exclusion (5), and their ability to undergo subsequent transformation when subjected to a mitogenic stimulus was assessed. Lymphocytes ($25–35 \times 10^6$), in a total volume of 2.0 ml of RPMI medium and 12.5% of human type AB serum, were incubated for 18 hr in the presence of 2.2 mg/ml of HAFP or HSA, with conditions identical to those used for lymphocyte cultures. A portion (4×10^6) of the lymphocytes was removed from the cell suspensions, spun down, and used for the determination of viability by the trypan blue method. The remainder of the cell suspension was diluted with 5 ml of Amos A gelatin (9) buffer and centrifuged at $150 \times g$ for 15 min. The cell buttons were washed once more with 7 ml of Amos buffer, resuspended in culture medium, and used for transformation studies.

The ability of HAFP to inhibit E-rosette formation by Ficoll-Hypaque-isolated human lymphocytes was determined by means of the E-rosette formation procedure, carried out in the presence of 2.5 mg/ml of HAFP . This procedure was similar to one already described (13), except that heat-inactivated human serum (type AB), previously absorbed with sheep red blood cells, was used in place of fetal calf serum.

In addition to the effect of HAFP in the mixed lymphocyte culture (MLC), the effect of HAFP on lymphocyte transformation by a nonhemagglutinating phytohemagglutinin (L-PHAP), concanavalin A (Con A), pokeweed mitogen (PWM), and rabbit anti-human thymocyte serum (ATS) was studied. The source and method of preparation of these mitogens have been described previously (5). The following amounts of mitogen were used in all experiments, unless otherwise indicated: L-PHAP, 10 μg; Con A, 100 μg; PWM, 0.08 μg; and ATS, 25 μl.

RESULTS

When unstimulated human lymphocytes were cultured in the

Abbreviations: HAFP, human alpha-fetoprotein; T-lymphocytes, thymus-derived lymphocytes; MLC, mixed lymphocyte culture; L-PHAP, nonhemagglutinating phytohemagglutinin; Con A, concanavalin A; PWM, pokeweed mitogen; ATS, rabbit anti-human thymocyte serum.

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† S. Yachnin, R. Hsu, R. Heinrikson, and J. B. Miller, "Studies on human alpha-fetoprotein: Isolation and characterization of monomeric and multimeric forms and amino-terminal sequence analysis," manuscript submitted.

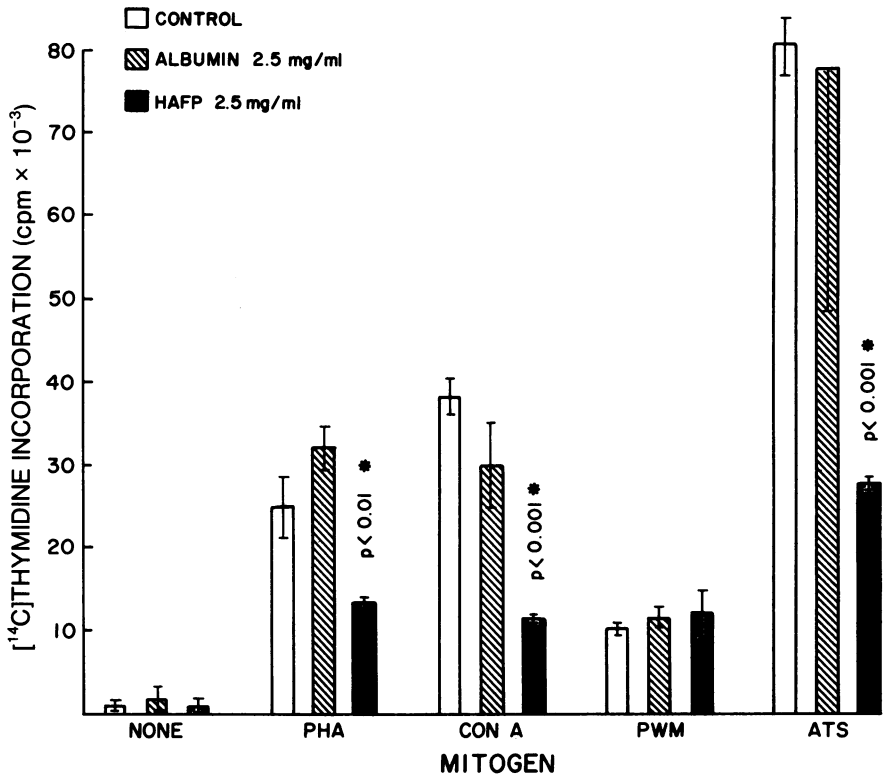


FIG. 1. Effect of HAFP (2.5 mg/ml) on mitogen-induced lymphocyte transformation. Brackets indicate ±SD. * = P values significant by comparison with control lymphocyte transformation.

presence of 1 mg/ml of HAFP for 90 hr, there was no inhibition of the incorporation of ^{14}C-labeled thymidine into the cells. At this concentration of HAFP, the mitogenic response of human lymphocytes to ATS (three experiments) was consistently inhibited (61.7, 52.3, and 26.3% inhibition), and the mitogenic activity of Con A was occasionally inhibited (one of three experiments; 63.6% inhibition). On two occasions, 1 mg/ml of HAFP failed to inhibit the lymphocyte response to L-PHAP or PWM.

At an HAFP concentration of 2.5 mg/ml, thymidine incorporation by resting lymphocytes, as well as the mitogenic response of human lymphocytes to PWM, remained unchanged; the lymphocyte responses to the other three mitogens, however, were significantly depressed (Fig. 1).

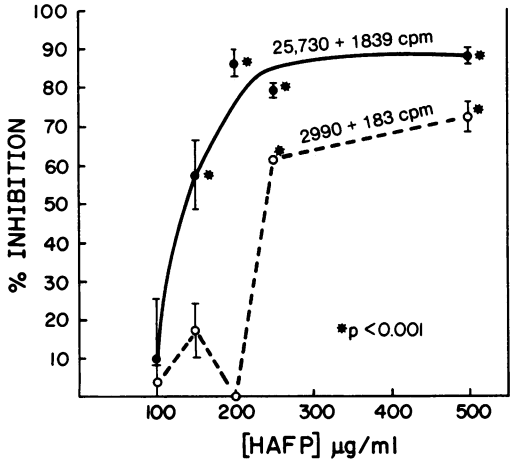


FIG. 2. Inhibition of the human one-way MLC by HAFP. O- - -O, Resting lymphocyte DNA synthesis; ●—●, MLC. The bar represents ±1 SD. The numbers shown adjacent to each plot represent the control values of [^{14}C]thymidine incorporation for resting and stimulated lymphocytes in the absence of HAFP addition.

When resting human lymphocytes isolated by the Ficoll–Hypaque technique were cultured in the presence of 200–500 µg/ml of HAFP for 138 hr, significant inhibition of thymidine incorporation by the cells was frequently noted. The presence of HAFP in the culture medium at similar concentrations also resulted in marked suppression of the human one-way MLC. The inhibitory effects of HAFP were no longer apparent at a concentration of 100 µg/ml (Fig. 2). The suppression of resting-lymphocyte DNA synthesis by HAFP was less than the suppression of the MLC-stimulated cells by the same HAFP concentration, and frequently marked suppression of MLC thymidine incorporation occurred at concentrations of HAFP that no longer suppressed resting-lymphocyte DNA synthesis.

Preincubation of lymphocytes in culture medium containing 2.2 mg/ml of HAFP for 18 hr, followed by washing, did not adversely affect the capability of the cells to take part in a one-way MLC, either as responding cells or as mitomycin-treated stimulating cells (Table 1). In addition, such exposure to HAFP did not result in any increase in cytotoxicity as measured by trypan blue exclusion.

Fetal HAFP (1 mg/ml) was substantially more effective in inhibiting both mitogen-induced and MLC-induced lymphocyte transformation than was the HAFP derived from the hepatoma patient (Table 2). In this experiment, fetal HAFP also profoundly inhibited resting-lymphocyte DNA synthesis at both 90 and 138 hr of incubation. Fetal HAFP was capable of inhibiting mitogen-induced lymphocyte transformation at concentrations as low as 80 µg/ml (Table 3). It should be noted that PWM-induced lymphocyte transformation was substantially suppressed at these concentrations of fetal HAFP, albeit to the least extent of the four mitogens tested; resting lymphocyte transformation was not affected.

Table 1. The effect of preexposure of human lymphocytes to human serum albumin or HAFP upon their subsequent behavior in MLC

Preexposure conditions	Thymidine incorporation (cpm)* Unstimulated† lymphocytes	One-way MLC	Trypan blue*‡ cytotoxicity (% dead cells)
Human serum albumin 2.2 mg/ml; 18 hr	3,767 ± 446	19,824 ± 2,181	28.4 ± 9.1 (n = 5)
HAFP 2.2 mg/ml; 18 hr	4,733 ± 1,237	23,522 ± 2,638	36 ± 7.6 (n = 5)

* No significant differences between HAFP and human serum albumin controls as determined by Student's t test. Results are mean value ± 1 SD. n refers to number of experiments.
† Responding cells only. The mitomycin-treated lymphocytes were also unaffected by HAFP preexposure, but incorporated much less isotope (approximately 150 cpm). Mitomycin-treated cells preexposed to either protein were equally effective as stimulating cells.
‡ Responding cells only.

The presence of 2.5 mg/ml of HAFP had no effect upon the capacity of human lymphocytes to form E-rosettes (Table 4).

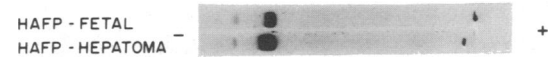
DISCUSSION

Our studies demonstrate that HAFP is capable of inhibiting *in vitro* transformation of human lymphocytes induced by phytomitogens, ATS, and allogeneic human lymphocytes. In this respect, the behavior of HAFP is similar to that which we have described for fetuin, a bovine alpha-fetoprotein (5); its inhibitory effects also resemble those of murine alpha-fetoprotein on murine lymphocyte transformation (7). Our observation on the relatively high concentrations of hepatoma HAFP (equivalent to those of fetuin) required for human lymphocyte suppression *in vitro* as opposed to murine alpha-fetoprotein, suggests that inhibition at low concentrations may not be a universal characteristic of this alpha-fetoprotein effect. The variability may be due to species differences, alterations of the protein during isolation, the presence or absence of small low-molecular-weight materials bound to the protein, or to special characteristics of HAFP derived from fetuses as opposed to hepatoma-bearing adults (see below).

Since HAFP is a glycoprotein (11), the possibility that it inhibits phytomitogen-induced lymphocyte transformation by association with the mitogenic agent must be considered (5, 14). We have not studied these possible interactions in detail, but others have described the binding of some HAFP molecules to Con A in free solution as well as to Con A immobilized on Sepharose beads (15). We have failed to demonstrate any precipitation when HAFP and L-PHAP or hemagglutinating phyto-hemagglutinin (H-PHAP) are allowed to diffuse toward one another in an Ouchterlony plate. As in the case of fetuin (5), the most compelling evidence against the possibility that HAFP competes with the responding lymphocyte for the mitogenic stimulus is that the most potent inhibitory effects of HAFP are manifest in the one-way MLC, where the mitogenic stimulus is due to the structural difference between allogeneic cell surface membranes. The failure of HAFP to cause nonspecific lymphocytotoxicity as measured by trypan blue exclusion following an 18 hr incubation suggests that nonspecific cell death also plays little or no role in HAFP inhibition of

Table 2. Comparison of the inhibition of lymphocyte DNA synthesis by hepatoma and fetal HAFP

Additions	Unstimulated lymphocytes cpm	% Inhibition	Con A 100 μg cpm	% Inhibition	ATS 25 μl cpm	% Inhibition	One-way MLC cpm	% Inhibition
None	2,830 ± 181	—	53,440 ± 7,020	—	35,800 ± 2,028	—	41,667 ± 5,173	—
Albumin 1 mg/ml	2,656 ± 366	6.2 (NS)	60,074 ± 28	0	43,098 ± 5,179	0	49,418 ± 4,514	0
Hepatoma HAFP 1 mg/ml	3,024 ± 296	0	52,335 ± 310	2.1 (NS)	26,395 ± 674	26.3 (P<0.01)	5,246 ± 478	874 (P<0.001)
Fetal HAFP 1 mg/ml	209 ± 55	96.2 (P<0.001)	0	100	45 ± 63	99.9 (P<0.001)	227 ± 20	99.5 (P<0.002)



Note that both HAFP preparations contain a small amount of HAFP dimer. The values for nonstimulated lymphocyte DNA synthesis in the MLC experiment are as follows: control, 11,740 ± 2,790 cpm; albumin, 8,279 ± 790 cpm (NS, not significant); hepatoma HAFP, 4,965 ± 190 cpm (P < 0.05); fetal HAFP, 121 ± 63 cpm (P < 0.002). All statistical comparisons are between cultures containing protein additions, and the phosphate-buffered saline controls.

Table 3. The inhibition of mitogen induced transformation by fetal HAFP

Mitogen	No added protein	Human serum albumin (80 μg/ml)			Fetal HAFP (80 μg/ml)		
	cpm	cpm	% Inhibition	(P value)	cpm	% Inhibition	(P value)
None	358 ± 36	375 ± 71		(NS)	333 ± 23		(NS)
L-PHAP	36,376 ± 2,494	35,567 ± 2,742	2.2	(NS)	7,458 ± 3,045	79.5	(<0.001)
Con A	64,934 ± 4,348	65,540 ± 4,444	0		9,421 ± 1,397	85.5	(<0.001)
PWM	18,811 ± 804	20,675 ± 1,665	0		6,702 ± 910	64.4	(<0.001)
ATS	68,002 ± 3,292	66,144 ± 8,615	2.7	(NS)	16,266 ± 886	76.1	(<0.001)

NS = not significant.

lymphocyte transformation. This conclusion is also borne out by the differential HAFP inhibition of lymphocytes stimulated by a variety of mitogens; if nonspecific cell death were the explanation of the inhibiting effect from HAFP, all mitogens (Con A, PWM, L-PHAP, and ATS) should have been affected equally. Further evidence for the lack of nonspecific HAFP lymphocytotoxicity is HAFP failure to inhibit the MLC when the cells involved were preincubated with HAFP for 18 hr and washed free of the protein prior to initiation of the MLC, although the concentrations of HAFP used during the preexposure were 5- to 10-fold higher than that required for inhibition when HAFP was present during the entire MLC incubation.

HAFP derived from the hepatoma patient does not adversely affect resting-lymphocyte DNA synthesis in short-term cultures of column-purified lymphocytes. However, significant depression of resting-lymphocyte thymidine incorporation by HAFP was usually observed in longer-term control cultures of Ficoll-Hypaque-isolated mononuclear cells during MLC experiments. The suppression of nonstimulated lymphocyte responses by HAFP occurs predominantly in longer-term lymphocyte cultures which are, by comparison with the short-term lymphocyte cultures prepared by nylon column-passage, proportionately richer in bone marrow derived lymphocytes (B-lymphocytes) (16). This suggests that HAFP may, in fact, suppress T-lymphocyte responses to autologous B-lymphocyte surface determinants (17). Such a conclusion would be justified by the results of our studies; human lymphocyte responses to mitogens including Con A, L-PHAP, ATS, and the MLC, all of which are primarily or exclusively due to T-lymphocyte cell proliferation (18–22), were suppressed by HAFP, whereas the mitogenic effect of PWM, an effective B-lymphocyte stimulus (18, 19, 22, 23), was relatively resistant to such inhibition. The suppression of PWM-induced lymphocyte transformation by fetal HAFP may be due in part to the fact that T-lymphocyte proliferation can be induced by PWM, and may be required to enhance B-lymphocyte responses to PWM. Thus, the immunosuppressive effects of HAFP are probably selective, and are directed primarily to the T-lymphocyte population; a similar conclusion has been reached by Tomasi and his coworkers regarding murine alpha-fetoprotein (6, 7). Despite the capacity of HAFP to inhibit T-lymphocyte response to mitogens, HAFP lacks the ability to inhibit E-rosette formation (24).

Table 4. The effect of HAFP on E-rosette formation by human lymphocytes

Additions	% E-rosette formation (±SD)
Phosphate-buffered saline control	61.4 ± 10.5
Human serum albumin 2.5 mg/ml	62.6 ± 8.1
HAFP 2.5 mg/ml	59.9 ± 4.6

The presence of HAFP or human serum albumin did not alter the results significantly. $n = 7$, number of experiments.

A marked difference was found between the capacities of fetal HAFP and hepatoma HAFP to inhibit lymphocyte responses. In addition, fetal HAFP more profoundly inhibited DNA synthesis by resting lymphocytes. One might argue that these differences reflect denaturation of hepatoma HAFP by our isolation procedures; however, precisely the same techniques were used for isolation of both proteins. Differences in primary amino acid sequence or in carbohydrate side chains could also be responsible for this difference in biologic potency (3, 25–27), but we could elicit no evidence for such alterations by electrophoretic behavior or immunologic characteristics† (28), and others have found the amino acid and sugar composition of fetal and hepatoma HAFP to be almost indistinguishable (11, 29). The possibility remains that the greater inhibitory effects of fetal versus hepatoma HAFP may be due to some low-molecular-weight molecule bound to fetal HAFP that emerges from a different biological environment and is peculiar to fetal metabolism. However, this putative substance would have remained bound to fetal HAFP at low pH (2.8); in addition, it could not be removed from the larger protein by molecular sieve chromatography or extensive dialysis, features that would make simple noncovalent binding less than likely. The marked difference in the biological potency of fetal and adult hepatoma HAFP remains to be determined.

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1. Adinolfi, A., Adinolfi, M. & Lessof, M. H. (1975) *J. Med. Genet.* **12**, 138–151.
2. Belanger, L., Hamel, D., Lachance, L., Dufour, D., Tremblay, M. & Gagnon, P. M. (1975) *Nature* **256**, 657–659.
3. Alpert, E., Drysdale, J. W., Isselbacher, K. J. & Schur, P. H. (1972) *J. Biol. Chem.* **247**, 3792–3798.
4. Nunez, M. E., Engelmann, F., Benassayag, C. & Jayle, M. M.-F. (1971) *C.R. Acad. Sci. Paris* **273**, 831–834.
5. Yachnin, S. (1975) *J. Exp. Med.* **141**, 242–256.
6. Murgita, R. A. & Tomasi, T. B., Jr. (1975) *J. Exp. Med.* **141**, 269–286.
7. Murgita, R. A. & Tomasi, T. B., Jr. (1975) *J. Exp. Med.* **141**,

440–452.
8. Allen, L. W., Svenson, R. H. & Yachnin, S. (1969) *Proc. Natl. Acad. Sci. USA* **63,** 334–341.
9. Yachnin, S., Allen, L. W., Baron, J. M. & Svenson, R. H. (1972) *Cell. Immunol.* **3,** 569–589.
10. Yachnin, S. (1972) *Clin. Exp. Immunol.* **11,** 109–124.
11. Hirai, H., Nishi, S., Watabe, H. & Tsukada, Y. (1973) *Alpha-Fetoprotein and Cancer,* Gann Monograph on Cancer Research No. 14, eds. Hirai, H. & Miyaji, T. (University of Tokyo Press, Tokyo), pp. 19–34.
12. Polet, H. & Spieber-Polet, H. (1975) *J. Exp. Med.* **142,** 949–959.
13. Braylan, R., Variakojis, D. & Yachnin, S. (1975) *Br. J. Haematol.* **31,** 553–564.
14. Yachnin, S. & Svenson, R. (1972) *Immunology* **22,** 871–883.
15. Smith, C. J. & Kelleher, P. C. (1973) *Biochim. Biophys. Acta* **317,** 231–235.
16. Greaves, M. F. & Brown, G. (1974) *J. Immunol.* **112,** 420–423.
17. Opelz, G., Kiuchi, M., Takasugi, M. & Terasaki, P. I. (1975) *J. Exp. Med.* **142,** 1327–1333.
18. Geha, R. S. & Merler, E. (1974) *Eur. J. Immunol.* **4,** 193–199.
19. Janossy, G. & Greaves, M. F. (1972) *Clin. Exp. Immunol.* **10,** 525–536.
20. Lohrmann, H. P., Novikovs, L. & Graw, R. G., Jr. (1974) *J. Exp. Med.* **139,** 1553–1567.
21. Häyry, P., Andersson, L. C., Nordling, S. & Virolainen, M. (1972) *Transplant. Rev.* **12,** 91–140.
22. Greaves, M., Janossy, G. & Doenhoff, M. (1974) *J. Exp. Med.* **140,** 1–18.
23. Parkhouse, R. M. E., Janossy, G. & Greaves, M. F. (1972) *Nature New Biol.* **235,** 21–22.
24. Gupta, S. & Siegal, F. P. (1975) *N. Engl. J. Med.* **293,** 302–303.
25. Gustine, D. L. & Zimmerman, E. F. (1973) *Biochem. J.* **132,** 541–551.
26. Zimmerman, E. F. & Madaffally, M. M. (1973) *Biochem. J.* **134,** 807–810.
27. Purves, L. R., van der Merwe, E. & Bersohn, I. (1970) *S. Afr. Med. J.* **44,** 1264–1268.
28. Adinolfi, A., Adinolfi, M. & Cohen, S. (1971) *Biochim. Biophys. Acta* **251,** 197–207.
29. Ruoslahti, E., Seppälä, M., Pihko, H. & Vuopio, P. (1971) *Int. J. Cancer* **8,** 283–288.
30. Yachnin, S. (1975) *Clin. Res.* **23,** 503A (abstr.).
31. Yachnin, S. (1975) *Blood* **46,** 1035 (abstr.).