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Inhibition of human lymphocyte transformation by human alpha-foetoprotein (HAFP); comparison of foetal and hepatoma HAFP and kinetic studies of *in vitro* immunosuppression

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SUMMARY

Five pure isolates of human alpha-foetoprotein (HAFP) from adults with tumours of the liver or stomach, as well as HAFP isolated from foetal liver, inhibit *in vitro* human lymphocyte transformation induced by phytomitogens, anti-human thymocyte serum, and the mixed lymphocyte culture. Foetal HAFP produces 50% inhibition at concentrations of 1–5 μg/ml. The HAFPs isolated from tumour-bearing adults are 1–3 orders of magnitude less potent (50% inhibition achieved at approximately 20, 130, 500, and 2000 μg/ml, respectively). In order to achieve maximum inhibition HAFP must be present at the time of mitogen addition; pre-exposure of lymphocytes to HAFP, followed by washing, does not result in lymphocyte suppression. The inhibiting effect of HAFP cannot be overcome by a ten-fold increase in mitogen concentration implying that HAFP does not act by simple competition with the lymphocyte membrane for the mitogen combining site. HAFP may play an immunoregulatory role during foetal development.

INTRODUCTION

Our laboratory has recently described the ability of human alpha-foetoprotein (HAFP) to inhibit the transformation of human lymphocytes *in vitro* by a variety of phytomitogens, rabbit antihuman thymocyte serum (ATS), and the mixed lymphocyte culture (MLC) (Yachnin, 1975a; Yachnin, 1975b; Yachnin, 1976; Yachnin *et al.*, 1976a). We noted that the HAFP derived from the serum and ascitic fluid of a hepatoma patient (McF.) was 1–2 orders of magnitude less potent in inhibiting these lymphocyte responses than was the HAFP derived from the serum of 15- to 20-week-old human foetal abortuses. Thus, in order to achieve >50% inhibition of human lymphocytes stimulated by the phytomitogens concanavalin A (Con A) and a non-haemagglutinating phytohaemagglutinin (L-PHAP), as well as ATS, concentrations of McF. HAFP 1000 μg/ml or greater were required; similar inhibition of human lymphocyte transformation by foetal HAFP required <80 μg/ml.

We have since had the opportunity to isolate HAFP from the serum and/or ascitic fluid of three more patients with hepatoma, one patient with gastric cancer and liver metastases, as well as from foetal liver. The hepatoma HAFP isolates display a wide range of potency with respect to the inhibition of human lymphocyte transformation. By far the most potent HAFP isolate is that derived from foetal liver which is 1–3 orders of magnitude more effective in inhibiting human lymphocyte transformation than the hepatoma HAFP preparations. The present report describes these differences, as well as further observations on the kinetics of HAFP inhibition of human lymphocyte reactivity.

MATERIALS AND METHODS

The method of isolation of HAFP has been described (Yachnin *et al.*, 1976a. In brief, it consists of passage of serum or ascitic fluid over an immunoadsorbent column of Sepharose 4B to which purified monospecific rabbit anti-HAFP antibody

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has been covalently linked by a cyanogen bromide reaction. After thorough washing with phosphate-buffered saline (PBS) pH 7·4, the bound HAFP is eluted with 0·1 M glycine buffer, pH 2·8, containing 0·5 M NaCl. The HAFP eluate is neutralized, concentrated, and passed over a second Sepharose 4B column to which the Na_2SO_4-precipitated globulin fraction of rabbit antihuman serum antibody has been attached. The concentrated fractions containing HAFP are then fractionated on a Sephadex G-150 column to remove the dimeric and trimeric forms of HAFP from HAFP monomer. Purified HAFP monomer is concentrated by Amicon ultrafiltration, sterilized by millipore filter passage, and stored frozen at −80°C in PBS at concentrations of 1–10 mg/ml.

HAFP was isolated from the liver of stillborn human abortuses of between 10–20-weeks gestation. The liver was homogenized in PBS by means of a Brinkman Polytron Tissue Homogenizer. After centrifugation at 20,000 *g* for 1 hr, the soluble liver extract was subjected to the procedures described above. All HAFP isolates were free of contamination by other human serum proteins as judged by analytical acrylamide gel electrophoresis and immunoelectrophoresis against a polyvalent rabbit antihuman serum antiserum. The yield of HAFP by these procedures was approximately 50%.

The techniques for the isolation of normal adult human peripheral blood lymphocytes, as well as methods for their culture have been previously described (Allen, Svenson & Yachnin, 1969; Yachnin, 1972a; Yachnin, 1975c). For MLC human mononuclear peripheral blood cells were isolated by means of a Ficoll–Hypaque gradient. For all other experiments, gravity sedimentation, osmotic shock lysis, centrifugation over a human plasma gradient, and nylon column passage were employed in lymphocyte isolation. The human lymphocytes so derived were >95% pure and were free of red blood cells and platelets. All lymphocyte cultures contained 1·5 to $2·0 \times 10^6$ lymphocytes in 1 ml RPMI+12·5% heat-inactivated AB serum. ^{14}[2-C]thymidine was added to mitogen stimulated cultures at 72 hr; the cells were harvested for liquid scintillation counting 16 hr later. MLC cultures were harvested at 136 hr, 16 hr following the addition of isotope. The source of mitogens, or their method of preparation, has been previously described (Allen, Svenson & Yachnin, 1969; Yachnin, 1972a; Yachnin, 1975c). Unless otherwise noted, the following amounts of mitogen were employed: Con A, 100 μg; L-PHAP, 10 μg; PWM, 0·08 μg; ATS, 25 μl.

RESULTS

The sources of the six HAFP isolates under investigation are shown in Table 1. These isolates, which were indistinguishable as judged by acrylamide gel disc electrophoresis and immunoelectrophoresis

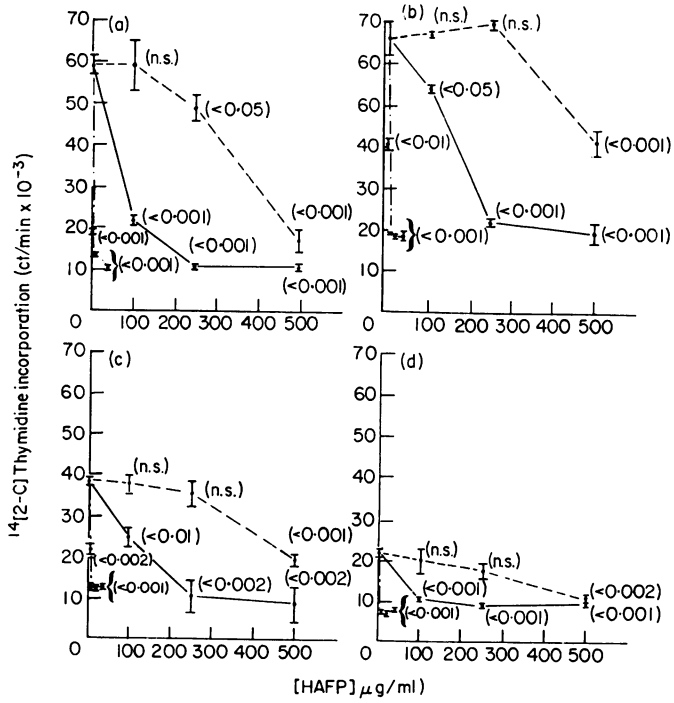

FIG. 1. The inhibition of lymphocyte transformation by varying doses of 3 HAFP isolates. A single preparation of lymphocytes was employed. Resting lymphocyte cultures incorporated 444±36 ct/min of the isotopic DNA precursor, and were not affected by the addition of any of the HAFP preparations. The brackets above and below each point represent ±1 s.d. The numbers in parentheses represent *P* values comparing each point with the control (no HAFP) mitogenic response. n.s. = Not significant (a), Con A; (b), ATS; (c), L-PHAP; (d), PWM. (- - - -), Ho HAFP; (——), Cr HAFP; (- · - ·), Foetal HAFP.

TABLE 1. A summary of the source of the various HAFP preparations

HAFP source	Age	Diagnosis	HAFP isolated from (μg/ml)
Cr.	64 years	Gastric Ca, Hepatic metastases	Serum (560 μg/ml)
McF.	61 years	Hepatoma	Serum (480 μg/ml) Ascites (250 μg/ml)
Ho.	72 years	Hepatoma	Ascites (100 μg/ml)
Od.	38 years	Hepatoma	Serum (255 μg/ml) Ascites (230 μg/ml)
Lu.	70 years	Hepatoma	Plasma (650 μg/ml)
Foetal liver	10–20 weeks	—	Homogenate (70 μg/ml) (90 ml)

against a monospecific rabbit anti-HAFP antiserum, nevertheless displayed wide variability in their potency as inhibitors of human lymphocyte transformation. Fig. 1 displays the results of a single experiment in which foetal HAFP was compared with two patient-derived preparations. Judging by the concentrations at which each HAFP preparation is capable of producing a 50% inhibition of lymphocyte transformation, foetal HAFP is 30–100 times more potent than Cr HAFP, which in turn is 3–5 times more potent than Ho HAFP. The data displayed in Table 2 are based on similar experiments and summarize our experience in comparing the potency of six HAFP isolates. Foetal HAFP is the most potent of our HAFP isolates. The HAFP preparations derived from the hepatoma and gastric cancer

TABLE 2. The concentration of various HAFP preparations required to inhibit lymphocyte transformation by various mitogens 50%*

	HAFP (μg/ml)					
Mitogen	Foetal	Lu.	Cr.	Ho.	McF.	Od.
L-PHAP	5	29 34	160 133	515	≅2000†	≅2000†
Con A	1·5	18 26 19	80 133	405 400 510	1000,† ≅2000	≅2000†
PWM	1	14 >100‡ (34·4%)	94	480	0§	0§
ATS	5	13 32 30	195	570 400 650	≅1000†	>2000‡ (19·3%)
MLC	2·8	n.d.	≅100†	n.d.	150, >300‡ (28%)	n.d.

n.d. = Not done.

* Values were obtained by experiments similar to those shown in Fig. 1, except as noted below.

† ≅ indicates that the inhibition of lymphocyte transformation at the concentration shown was ±1 s.d. of 50% inhibition. Higher concentrations were not tested.

‡ The percentage inhibition at the concentration of HAFP given is shown in parentheses. Higher concentrations were not tested.

§ No inhibition was noted at 2000–2500 μg HAFP/ml.

TABLE 3. The effect of pre-addition of HAFP for 18 hr, with or without washing, upon lymphocyte transformation

	HAFP and/or mitogen added T_o		Saline or HAFP added T_o, mitogen added 18 hr		Saline or HAFP added T_o, wash 18 hr, add mitogen	
Mitogen	ct/min±s.d.	Percentage inhibition (*P* value)	ct/min±s.d.	Percentage inhibition (*P* value)	ct/min±s.d.	Percentage inhibition (*P* value)
None						
Control	1146±110	—	2353±274	—	1326±208	—
HAFP	735±2	35·9 (<0·02)	614±18	73·9 (<0·01)	1191±159	10·2 (n.s.)
LPHAP						
Control	59556±4619	—	44107±3773	—	40040±5894	—
+HAFP	13128±260	78 (<0·001)	17680±73	59 (<0·01)	38088±3943	4·9 (n.s.)
Con A						
Control	81303±5156	—	60162±12235	—	46026±3663	—
+HAFP	18172±162	77·6 (<0·001)	15846±110	73·7 (<0·02)	51159±636	0 (n.s.)
ATS						
Control	70705±288	—	60195±936	—	61786±4637	—
+HAFP	19596±895	72·3 (<0·001)	19853±253	67 (<0·001)	68494±6293	0 (n.s.)

n.s. = Not significant.

A single preparation of lymphocytes was used throughout. All cultures were harvested 88 hr after addition of mitogen; ^{14}[2-C]thymidine was added at 72 hr. Wherever indicated, cultures received 500 μg/ml Cr HAFP.

patients' body fluids display a wide range of effectiveness in inhibiting lymphocyte transformation; two of the latter (McF. and Od.) are approximately 3 orders of magnitude less effective than foetal HAFP.

We have previously reported that pre-exposure to HAFP for 18 hr, followed by washing, did not impair the ability of lymphocytes to take part in a MLC. (Yachnin, 1976). The experiment shown in Table 3 indicates that the same is true for phytomitogen and ATS-induced lymphocyte responses. In addition, allowing lymphocytes to remain in contact with HAFP for 18 hr, followed by mitogen addition

TABLE 4. The effect of adding foetal HAFP to lymphocyte cultures at varying times after mitogen stimulation*

	Con A		ATS	
Culture description	ct/min±s.d.	Percentage inhibition (*P* value)	ct/min±s.d.	Percentage inhibition (*P* value)
T_o Control (No HAFP)	53051±2620	—	38484±2279	—
8 μg HAFP, To	10679±36	79·8 (<0·001)	14655±111	61·9 (<0·001)
8 μg HAFP, 24 hr	20100±544	62·1 (<0·001)	24440±1657	36·5 (<0·002)
8 μg HAFP, 48 hr	42872±2707	19·2 (<0·02)	37445±1020	2·7 (n.s.)
8 μg HAFP, 72 hr	52694±2762	0·7 (n.s.)	38392±1257	0·2 (n.s.)

n.s. = Not significant.

* Mitogens were added at T_o; ^{14}[2-C]thymidine was added at 72 hr, and the cultures were all harvested 16 hr later. Non-stimulated lymphocyte DNA synthesis (933 ct/min) was not significantly inhibited by the presence of HAFP.

without washing, did not increase the efficacy of HAFP inhibition of lymphocyte DNA synthesis. HAFP is most effective in inhibiting lymphocyte transformation if it is present in the culture medium at the time of mitogen addition. Addition of HAFP to cultures at 24-hr intervals following mitogen stimulation results in a progressive loss of its inhibitory action; 48 hr after initiation of the lymphocyte cultures, little or no inhibition occurs. Addition of HAFP at 72 hr, together with the isotopic DNA precursor, does not alter DNA synthesis at all during the subsequent 16 hr (Table 4).

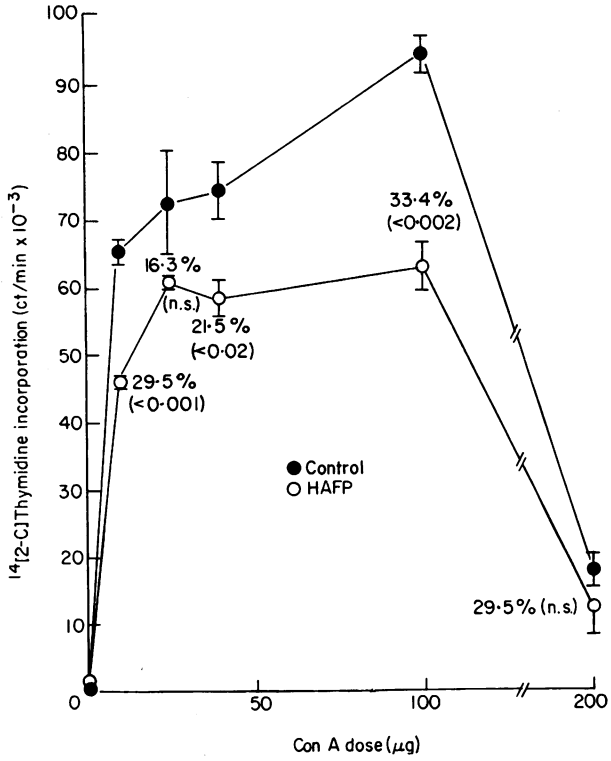

FIG. 2. The effect of increasing doses of mitogen on the inhibition of lymphocyte transformation by HAFP. HAFP (Cr.), 100 μg/ml, was used in HAFP-containing cultures. The percentage inhibition of lymphocyte transformation together with *P* values are shown for each mitogen dose. n.s. = Not significant. (○), HAFP; (●); control.

In our previous studies on the immunosuppressive effects of foetuin (a bovine AFP) (Yachnin, 1975c) and HAFP (Yachnin, 1976), we concluded that these proteins did not inhibit lymphocyte responses to mitogens by combining with the mitogens and preventing their attachment to the lymphocyte membrane. Further reinforcement of this conclusion can be seen in the experiment shown in Fig. 2. Inhibition of the lymphocyte response to Con A is constant over a wide dose range of the mitogen and cannot be abolished by a ten-fold increase in mitogen dose from 10–100 μg, as would be expected if simple competition with the lymphocyte membrane for the mitogen combining sites were the mechanism.

The ability of HAFP to inhibit lymphocyte transformation is not affected by heating at 60°C for 1 hr, but is completely abolished by heating at 80°C for 1 hr.

DISCUSSION

Until recently, little has been known of the biological role of the alpha-foetoproteins, although they have been regarded as an embryonic serum albumin (Belanger *et al.*, 1975; Alpert *et al.*, 1972), and have been shown to possess estrogen hormone-binding properties (Nunez *et al.*, 1971). Our report on the inhibition of human lymphocyte transformation by foetuin (Yachnin, 1975c), and the simultaneous observations by Tomasi and his co-workers that murine alpha-foetoprotein suppresses murine lymphocyte responses *in vitro* to both mitogenic and antigenic stimuli (Murgita & Tomasi, 1975a; Murgita & Tomasi, 1975b), suggest an immunoregulatory role for alpha-foetoproteins during foetal development.

The present report confirms our earlier observations on the greater potency of foetal HAFP as a suppressor of lymphocyte responses when compared with HAFP derived from patients with hepatoma (Yachnin, 1975a; Yachnin, 1975b; Yachnin, 1976; Yachnin *et al.*, 1976a). In addition it emphasizes that

such variability in immunosuppressive potency is not limited to a distinction between foetal HAFP and hepatoma HAFP, but can be seen within hepatoma HAFP isolates as a class as well, wherein a 2 order of magnitude difference can be easily demonstrated. While we could elicit no differences in the various HAFP preparations by the techniques of polyacrylamide gel electrophoresis or immunoelectrophoresis, others have described microheterogeneity in HAFP (Alpert *et al.*, 1972; Purves, van der Merwe & Bersohn, 1970) and murine AFP preparations (Gustine & Zimmerman, 1973) by extended electrophoresis in agarose or starch gels, which could play a role in the variability seen in regard to lymphocyte suppression. The molecular basis for the wide variations in the biological properties of HAFP isolates is currently under investigation in our laboratory.

We have previously demonstrated that preincubation of HAFP with human lymphocytes, followed by washing, does not impair the ability of such cells to take part in a MLC mitogenic response (Yachnin, 1976). The present report emphasizes that human lymphocytes treated in a similar fashion are also totally unimpaired in their response to other mitogenic stimuli as well. In this regard, HAFP differs from murine AFP, since the latter was capable of inhibiting the primary immune response of mouse splenocytes *in vitro* if the cells were pre-exposed to it for 4–8 hr and then washed prior to initiation of the cultures (Murgita & Tomasi, 1975a). No information is available regarding the effects of pre-exposure of murine lymphocytes to murine AFP on their subsequent response to mitogens or the MLC. Thus, if HAFP acts to inhibit human lymphocyte responses by virtue of its ability to bind to a subpopulation of these cells, as has been suggested for murine AFP-lymphocyte suppression (Dattwyler, Murgita & Tomasi, 1975), such binding must be one of very low affinity, and results in no irreversible suppressive effects on the subsequent capacity of the human lymphocyte to respond to a variety of mitogenic stimuli. HAFP is, in this regard, analogous to the immunoregulatory alpha-globulin (Cooperband *et al.*, 1972) and to human chorionic gonadotropin (Adcock *et al.*, 1973), since both of the latter must also be present throughout the duration of lymphocyte culture in order to express their ability to inhibit lymphocyte mitogenic responses.

While pre-exposure of lymphocytes to HAFP for 18 hr does not enhance the ability of the protein to inhibit subsequent mitogen responses beyond that achieved by simultaneous addition of HAFP and mitogen, a progressive decrease in the inhibitory effects of HAFP on lymphocyte responses is seen if HAFP addition to the cultures is delayed for 24 hr or longer. By the time DNA synthesis is well established (48 and 72 hr), little or no inhibition of DNA precursor incorporation into lymphocyte DNA can be achieved by addition of HAFP to the cultures. The lack of reversibility of HAFP lymphocyte suppression to Con A by increasing doses of the mitogen speaks against simple competition by HAFP for the mitogen combining site. Whether HAFP acts to prevent access of mitogens to the lymphocyte cell membrane by a 'halo' or 'blindfolding' mechanism (Yachnin, 1972b; Yachnin, 1975c) or whether it interferes with the generation of a cell membrane signal necessary for the commitment of the lymphocyte to DNA synthesis and mitosis following mitogen attachment, remains to be clarified. By whatever mechanism HAFP achieves its immunosuppressive effects, its potential role during foetal life in suppressing forbidden lymphocytes clones, and in maintaining the foetus in an immunologically hostile environment, is of obvious importance.

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