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In vitro and *in vivo* analyses of a genetically-restricted antigen specific factor from mixed cell cultures of macrophage, T and B lymphocytes

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ABSTRACT

An immunostimulatory factor was identified 5o be secreted by antigen-pulsed macrophages. This factor was able to induce the generation of antigen specific T helper lymphocytes *in vitro* as well as *in vivo*. Further *in vitro* experiments testing for the genetic restriction of this factor indicated that it is a genetically-restricted antigen specific factor (ASF). The Cunningham plaque assay was used to quantify the generation of T helper lymphocytes by measuring the number of plaque forming cells after sequential incubations of antigen-pulsed macrophages with T lymphocytes, and then spleen cells, and finally the TNP-coated sheep red blood cells.

Key words: *Antigen specific factor, genetic restriction, macrophages, lymphocytes, antigen presentation.*

INTRODUCTION

It has been established that the classic antigen presenting cell is macrophage whose participation is required for the activation of many T lymphocyte functions. The importance of direct macrophage-lymphocyte contact in lymphocyte activation has been emphasized by several investigators[1, 2, 3, 4]. In the present study, we have examined the interactions between T cells and antigen-pulsed maorophages in order to test whether direct centact between the two cell types is an obligatory s step for the induction of antigen specific helper T *cells in vitro*, or additional mechanism also exists which is mediated by soluble immunostimulatory factors from maorophages.

We have also examined whether this induction of helper T cells (T) by a putative macrophage factor, is genetically restricted as is the activation of specific immune T cells in the presence of antigen-bearing macrophages [5, 6, 7, 8]. Finally we have tested the genuine existence of ASF by the induction of helper T cells *in vivo*.

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MATERIALS AND METHODS

Animals

All mice were bred at the Laboratory Animal Unit, University of Hong Kong. The mouse strain used for the experiments was C57BL/6 unless specified otherwise. The mice were males aged between 9 to 12 weeks.

Antigens

The antigen used was horse spleen ferritin (HSF, Miles Laboratories) unless specified otherwise. Other antigens used were keyhole limpet hemocyanin (KLH, Sigma), horseradish peroxidase (HRP, Sigma) and bovine serum albumin (BSA, Sigma).

Preparation of macrophages

Mouse peritoneal macrophages were prepared according to the method of Beller and Unanue [9]. The peritoneal exudate cells were plated on either coverslips or culture flasks (Falcon), cultured for 2 hr at 37℃, and thereafter the nonadherent cells were removed by washing. Some preparations were treated with anti-Thy 1.2 (generous gift from N. I. H. , U. S. A.) and complement to remove any contaminating T cells which might absorb some of the factors produced by the maorophages.

Preparation of T lymphocytes

Mouse splenic T lymphocytes were isolated by nylon wool filtration based on the procedure described by Julius *et al*. [101. The viability of cells was greater than 95% as judged by dye exclusion ability. The proportion of surface membrane Ig+ cells in this preparation was less than 3% as detected by staining with FITC-anti-mouse Ig. The percentage of Mac-2^+ cells was less than 0.1% as detected by monoclonal antibody against routine maorophage surface Mac-2 antigen (from Boehringer Mannheim Biochemioals, U.S.A.).

Culture conditions

Maorophages were pulsed with antigen (20μg/ml) for 1 hr after which the cells wore washed 4 times and then cultured either alone or together with T cells. To obtain a factor the antigen-pulsed maorophages were cultured in serum-free RPMI 1640 (Flow Laboratories) containing 2.2 g/l of sodium bieabonate, 5 mM HEPES (Research Organics), 2m*M* L-glutamine (GIBCO), 100 units/ml Penicillin and 100μg/ml Streptomycin (GIBCO). After 4 days, the culture supernatant which contained the factor was harvested and ultrafiltered with PM 10 membrane (Amieon Corporation) to remove dialysable substances. This crude supernatant was millipore-filtered before and after the PM 10 membrane ultrafiltration.

Lymphocytes were cultured in a similar medium with the additional oonstituents of 10% heat-inactivated fetal bovine serum (FBS, GIBC0) and 50μM 2-mercaptoethanol (Sigma).

Binding of T cells to macrophage

Normal and antigen-treated (obtained from mice each injected intraperitoneally with 100μg HSF in 10% peptone 3 days before sacrifice) macrophages wore co-cultured with T cells prepared from HSF-primed or unprimed mice for 3.5 hr at 37℃. The unbound T cells were then washed away gently. The monolayers of macrophages with the bound T cells were fixed, stained (Wright stain) and counted.

Induction of helper T cells and T-B cooperation

Induction of antigen specific helper T cells was achieved by incubating nylon wool purified T cells with antigen-pulsed macrophages or the factor without any macrophags. After 4 days, a small number of viable cells (5×10^4) were washed and then added to cultures of spleen cells (1×10^6 cells per culture) which provided the B]ymphocytes. For each T cell sample, quadruplicate cooperabion culbures were set up. These cells were co-cultured in the presence of trinitrophenyl (TNP)-carrier (0.1μg/ml) for 4 days after which the TNP specific plaque forming cells (PFC) were assayed with TNP-sheep red blood cells (TNP-SRBC) and complement.

PFC assay

The hemolytic PFG assay described by Cunningham and Szenberg [11] was adopted. The lymphocytes from the cooperation cultures were washed and mixed with TNP-SRBC and guinea pig serum which served as a source of complomen‌t Rabbit anti-mouse IgG (Sigma) was included in some assays to test for the presence of, if any, IgG-producing B cells. The mixture was introduced into duplicate shallow chambers which were sealed with a molten mixture of paraffin wax and petroleum jelly (1:1) and incubated at 37℃ for 3-4 hour. The number of PFG per chamber was determined by scanning the entire chamber under the microscope, using the 10× objective.

Induction of antigen-specific helper T cells in rive

Mice were injected intraperitoneally 3 times with either 0.5ml of saline or factor (10 μg/ml) per animal. No adjuvant was used in any of those injections. T cells were isolated from the spleens of these mice 1 wk after the 3rd dose of injection and used for cooperation. Four days later, the PFG assay was done.

Trinitrophenylation of proteins

Soluble proteins were trinitrophenylated by the method of Rittenberg and Amkraut [12] using 2, 4, 6-trinitrobenzene sulfonic acid (Nutritional Biochemicals Corporation). The solution of coupled protein was dialysed and then passed through a column of Sephadox G25 (Pharmacia) to remove the side-reaction products. The coloured fraction in the void volume was collected and concentrated by PM 10 membrane ulrafiltration.

Protein determination

The amount of proteins in samples of the factor was determined by the method of Lowry *et al*. [13] using BSA as the standard.

RESULTS

Binding of T cells to macrophages.

When mouse splenic T cells were added to peritoneal maorophages, some of these T cells became bound to the macrophages whether one or both cell types had previously encountered the antigen (HSF) or not. Tab. 1 shows that the antigen nonspecific binding between normal T cells and peritoneal maorophages was low with only 5% bound maorophages and wibh an average of 2.3 T cells per bound maorophage. Binding of HSF-primed T cells to normal maorophages was insignificantly higher than that of normal T cells. However, a significantly higher percentage (10.5 %) of bound macrophages with an average of 3 T cells per bound macrophage was observed when macrophages but not T cells had previously been treated with the antigen. These results indicate that encounter of antigen by macrophages prior to the binding experiment was more important than that by T cells for the physical interaction between macrophages and T cells. Best physical interaction was observed if both cell types had been exposed to the same antigen before the experiment.

Tab. 1 Einding of T cells to macrophages

Macrophages (5×10^5)	T Cells (3×10^6)	% of bound[e] macrophages	No. of T cells[f] Bound Mϕ
Control[a)]	Control[c)]	5.0±0.8	2.3±0.8
Control[a)]	HSF-primed[d)]	5.7±0.7	2.6±0.6
HSF-treated[b)]	Control	10.5±0.5	3.0±0.2
HSF-treated	HSF-primed	14.3±0.7	3.0±0.2

a) Peritoneal Mϕ obtained from mice injected with 10% peptone.
b) As a) except injected with HSF (100μg/mouse) in 10% peptone.
c) T cells prepared from spleens of normal mice.
d) T cells prepared from spleens of HSF-primed mice.
e) The mean percentage of .T cell-bound Mϕ per 200Mϕ and the S. E. M. for 3 separate experiments(n=4).
f) The mean number of T cells per bound Mϕ in 20 bound Mϕ and the S. E. M. (n=4).

In vitro induction of helper T cells

Tab. 2 shows that only the antigen-pulsed macrophages were capable of inducing helper T cells in macrophage-T cell co-cultures. Macrophages pulsed with increased amounts of antigen were better antigen-presenting cells than those treated with lower amounts of antigen. Although the response is lower, helper T cells could also be generated when the antigen-pulsed macrophages and T cells were co-cultured without direct physical contact. This result suggests that direct macrophage-T cell contact was important but notobligatory for the induction of antigen specific helper T cells. Furthermore, it also indicates that certain immunosbimulatory factor produced by the antigen-pulsed macrophages was responsible for the induction of helper T cells in these cultures since antigen specific helper T cells were not obtained from cultures in which T cells were cultured alone or with unpulsed macrophages.

Induction of helper T cells by macrophage-derived factor

As shown in Tab. 3 supernatant harvested from cultures of antigen-pulsed maorophages

Tab. 2 Generation of helper cells from interaction of antigen-pulsed macrophages and cells[a)]

1st Culture T_H induction		2nd Culture T-B cooperation			Anti-TNP response
Macrophages (5×10^5)	T cells (5×10^6)	T/T_H cells transferred (5×10^4)	Spleen cells added $(1\times10)^6$	Challenge (0.1μg/ml)	PFC[b)]
-	-	-	+	TNP-HSF	60±12
-	+	+	+	TNP-HSF	30±10[d)]
unpulsed	+	+	+	TNP-HSF	55±13
HSF-pulsed (10μg/ml)	+	+	+	TNP-HSF	205±29[e)]
HSF-pulsed (20μg/ml)	+	+	+	TNP-HSF	275±25
HSF-pulsed (30μg/ml)	+	+	+	TNP-HSF	305±62
HSF-pulsed (10μg/ml)[C)]	+	+	+	TNP-HSF	165±59[f)]

a) T cells were co-cultured with macrophages which had either been pulsed or unpulsed with HSF. Four days later, the T cells were transferred to cultures of spleen cells. The generation of TH was measared by the cooperation between T and B cells in the presence of TNP-carrier and subsequently by the anti-TNP PFC assay.
b) The mean PFG number per culture and the S. E. M.
c) The maerophages which adhered to a coverslip were place on top of a grid after the T cells had settled onto the bottom of the culture vessel so that there was no direct contact between these two cell types.
d) Used as control for calculation of statistics by Student's t-test:
e) $p<0.01$, f) $p<0.01$.

Tab. 3 Induction of T_H by ASF[a)]

1st Culture T_H induction		2nd Culture T-B cooperation			Anti-TNP response
ASF	T cells (5×10^6)	T/T_H cells transferred (5×10^4)	Spleen cells added $(1\times10)^6$	Challenge (0.1μg/ml)	PFC[b)]
-	-	-	+	TNP-HSF	63±19
-	+	+	+	TNP-HSF	40±14[d)]
ASF_{KLH}[C)]	+	+	+	TNP-HSF	355±39[e)]
ASF_{KLH}	+	+	+	TNP-HSF	15±5
-	+	+	+	TNP-HSF	15±5
-	+	+	+	TNP-HSF	15±5
ASF_{HSF}	+	+	+	TNP-HSF	15±5
ASF_{HSF}	+	+	+	TNP-HSF	15±5
ASF_{KLH}	+	+	+	TNP-HSF	15±5
ASF_{KLH}	+	+	+	TNP-HSF	15±5
ASF_{KLH}	+	+	+	TNP-HSF	15±5
ASF_{KLH}	+	+	+	TNP-HSF	15±5
ASF_{KLH}	+	+	+	TNP-HSF	15±5

a) ASF harvested item antigen-pulsed macrophage cultures was introduced into T cell cultures. The generation of TH was measured as described in footnote a) of Table 2.
b) The mean PF0 number per culture and the S. E. ~f.
c) ASF from macrophages pulsed with KLH.
d) Used as controls for calculation of statistics by Student's t-lest:
e) $p<0.01$, f) $p<0.01$, g) $p<0.01$.

was capable of inducing helper T cells in the absence of macrophages or any additional antigen. These helper T cells were antigen specific because the T-B cooperation cultures yielded high number of PFC only if challenged with TNP conjugated So She antigen used for obtaining the factor. Similar results were obtained for all three soluble antigens studied. We named this macrophage-derived immunostimulatory moiety antigen-specific factor or ASF.

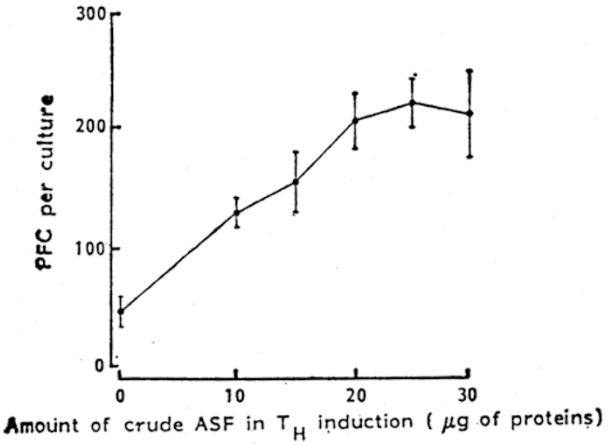

Fig. 1 Effect of graded amounts of ASF on helper cel] induction. Various amounts of crude ASF were added to cultures of T cells. The generation of TH was measured as described in footnote a) of Table 2.Each result represents the mean+S.E.M. of quadruplicate cu]tures.

Fig. 1 represents the dose response curve of ASF to determine the optimal concentration for induetion of helper T cells. The graph shows that this optimum was about 20-30 μg of crude ASF (PM 10 membrane ultrafilltered macrophage culture supernatant). The difference between 0 μg and 10 μg of crude ASF and that bebween 10 μg and 20 μg were significant, $p<0.01$ and $p<0.05$ respectively.

Speciticity of antibody forming cells generated from T-B cooperation

In order to demonstrate that the antibody forming cells generated from T-B cooperation were TNP specific, TNP-BSA or normal SRBC were used in some assay chambers. Tab. 4 shows that the excess TNP-BSA present in the assay completely inhibited plaque formabion probably by competition for TNP specific antibodies with the TNP-SRBC. A second teat for TNP specificity was used to make sure that the excess TNP-BSA did not inhibit plaque formation by interfering with the functions of antibody-forming cells. The use of normal SRBC instead of TNP-SRBC in the assay yielded negligible number of PFC. This excludes the possibility that these cells produced cytotoxic substances which were responsible for

Tab. 4 Specificity of an~iboay forming cells generaf~l from T-B ccopera~ion

Assay conditions	PFC+S. E. M.
Control[a)]	25±8[f)]
Normal[b)]	150±14[g)]
Normal-I-TNP-BSA added to PFG assay[c)]	17±8
Normal except SRBC unhaptenated[d)]	4±4
Normal + Rb anti-MlgG added to PFC assay[e)]	183 ± 40[h)]

a) No T cells transferred from first culture into second culture.
b) The generation of T_H was measured in the usual manner (see footnote a)of Table 2).
c) TNP-SRI C were mixed with competing TNP-BSA(0.11 mg/chamber) before used for PFC assay.
d) SRBC used in PFC assay were unhaptenated.
e) Rabbit avti-mouse IgG developing serum (4 μl/chamber)was included in the assay mixture.
f) Used as control for calculation of statistics by Student's t-test.
g) $p<0.01$.
h) Not significant compared with normal assay condition.

plaque formation. The requirement for haptenated SRBC in the formation of hemolytic plaques implies that the antibodies produced were happen specific.

The addition of rabbit anti-mouse IgG developing serum to the assay mixtmre did not yield more hemolytic plaques than the direct plaque assay. This indicates that only IgM and no IgG antibody-forming cells were found in our cultures in which unprimed B cells were employed.

Inability of ASF from allogeneic macrophages to facilitate inductioiz of helper T cells

If the strain of mice used for the preparation of T cells was different from that used for obtaining ASF, then only control levels of PF0 were obtained as illustrated in Tab. 5 The requirement for syngeneic macrophages and T cells suggests that helper T cell induction by ASP is likely yo be genetically restricted.

Tab. 5 Inability of ASF from allogeneic macrophages to t~acilitate TH induction

1st Culture T_H induction		2nd Culture T-B cooperation			Anti-TNP response
Source of ASF_{HSF}[a]	Source of T cells (5×10^6)	T/T_H cells cells added (5×10^4)	Spleen cells added (1×10^6)	Challenge ($0.1\mu g$/ml)	PFC[b]
ICR(out-bred)	ICR	+	+	TNP-HSF	18±9
–	C57BL/6	+	+	TNP-HSF	39±11[c]
CBA/N(H-2^k)	C57BL/6	+	+	TNP-HSF	29±12
BALB/c(H-2^d)	C57BL/6	+	+	TNP-HSF	25±15
C57BL/6(H-2^b)	C57BL/6	+	+	TNP-HSF	196±19[d]
–	BALB/c	+	+	TNP-HSF	25±12[c]
C57BL/6(H-2^b)	BALB/c	+	+	TNP-HSF	21±9
BALB/c(H-2^d)	BALB/c	+	+	TNP-HSF	139±18[e]

a) The strains of mice from which macrophages were isolated, pulsed and cultured to obtain ASF. The H-2 haplotypes of the different strains were given in parentheses.
b) The mean PFG number per culture and the S. E. M.
c) Used as control for calculation of statistics by Students's t-test:
d) $p<0.01$, e) $p<0.01$.

Tab. 6 *In vivo* induction of T_H by injection of ASF

T-B cooperation			Anti-TNP response
Source of T cells (5×10^4)	Spleen cells added (1×10^6)	Challenge ($0.1\mu g$/ml)	PFC[a]
Mice injected with saline	+	TNP-HSF	44±12[b]
Mice injected with ASF_{HSF}	+	TNP-HSF	172±19[c]
Mice injected with ASF_{HSF}	+	TNP-BSA	15±10
Normal mice	+	TNP-HSF	31±8
–	+	TNP-HSF	39±15

a) The mean PFG number per culture and the S. E. M.
b) Used as control for calculation of statistics by Students's t-test:
c) $p<0.01$.

In vice induction of helper T cells

So far, activity of ASF appears to be one of the mechanisms of T cell activation in vitro. However, these studies on ASF would bear much more significance if ASF can be shown to be active in vivo as well. Tab. 6 shows that T cells isolated from mice injected with ASF indeed contained antigen specific helper T cells. In addition, increased number of such T cells introduced into T-B cooperation cultures resulted in greater number of PFC (Fig. 2).

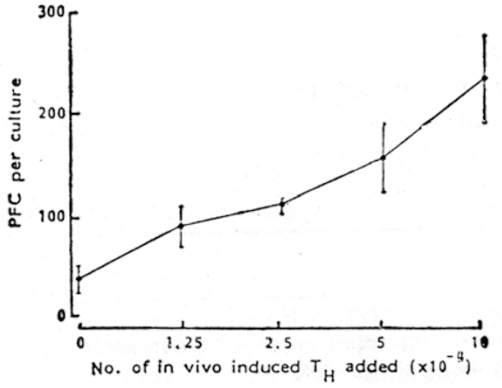

Fig. 2 Effect of graded numbers of *in vivo* induced TH on geneation of PFC in T-B cooperation. Various numbers of *in vivo* induced TH were used in T-B cooperation cultures. Four days later, the PFC assay was performed. Each result represents the mean' S. E. M. for quadruplicate cultures.

DISCUSSION

In the macrophage-T binding experiments, delayed fixation and scoring (t = 5-24 hr) yielded decreasing number of lymphocytes bound to macrophages (data not shown). This was probably due to dissociation of lymphocytes from the macrophages caused primarily by the decay of antigen-induced changes in macrophages as reported by Ben-Sasson *et al*. [14]. This dissociation should be important in an immune response *in vivo*. After interaction with macrophages, it is desirable for the T cells to migrate to other sites within the lymphoid tissue in order to interact with B cells and other T cell subsets.

The physical interaction between macrophages and T cells represents an example of cell cooperation in immune responses. There is considerable evidence which suggests that this interaction is necessary for T cell stimulation [15, 2, 3, 4] but definitive evidence is lacking. Our results show that direct physical interaction between these two cell types is important but not obligatory for the generation of helper T cells. The results in Tab. 3 clearly indicate that ASF harvested from antigen-pulsed macrophage cultures is capable of inducing antigen specific helper T cells in vitro in the absence of macrophages or any additional antigen.

The importance of direct macrophage-lymphocyte contact in antigen presentation and the involvement of soluble immunostimulatory factors like ASF are not as contradictory as they may first appear. The two modes of antigen presentation are not mutually exclusive. The action of soluble immunostimulatory factors may be augmented by the development of

physical contact between macrophages and T cells. Furthermore, macrophage-lymphocyte clusters may provide a focus for and thus enhance T-B cooperation [16].

There are results reported by several other groups which are analogous to ours [17, 18, 19]. These provide strong support for the genuine existence and importance of immunostimulatory factors like ASF. These ASF-like factors were given different names by different investigators: genetically related factor (GRF) [17], In-positive moiety (IPM) [18] and In-containing antigen complex (IAC) [19]. Apparently, it will be worthy to test whether all these immunostimulatory factors are in fact the same factor.

We have attempted to purify ASF and characterize it. Preliminary findings showed that ASF is a complex of processed antigen fragment and an I-region-defined antigen (In) moiety (unpublished results). This is consistent with the reports that presentation of antigen is in the context of an Ia complex [17, 18, 19, 20, 21].

Our observation that induction of helper T cells by ASF appears to be genetically restricted is also consistent with the findings reported by various groups [17, 18, 19, 7, 8]. Because of the difficulty in the availability of different inbred mouse strains in Hong Kong, we were unable to map the histocompatibility requirement for macrophage-T or ASF-T interaction to any particular region of the major histocompatibilify complex.

Although the antigen-presenting role played by macrophages in the generation of T cell immune responses has boon established, neither the pathway of antigen through the cell nor the precise biochemical events in antigen processing have been elucidated. At present, we are attempting to follow the intracellular fate of an antigen subsequent to endocytosis by macrophages. HSF was chosen to be the antigen because its electron dense property enables us to identify it easily in an electron micrograph. Even if this antigen processing pathway can be worked out, the question whether intracellular antigen processing by antigen-presenting cell is obligatory for presentation of antigen has yet to be solved; because nonphagocytic dendritic cells which hardly process any antigen have been. reported to be very potent antigen presenting cells [22, 23, 24]. Furthermore, extracellular (cell-flee) antigen processing has been observed [25].

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