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In vitro Induction of primary antibody responses to particulate and soluble protein antigens in T cell-replaced murine spleen cell cultures

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ABSTRACT

Specific antibody responses could be induced in serum, free condition. Specific anti-SRBC or anti-SRBC ghost antibody were induced from anti-Thy treated (T-depleted) murine spleen cells in serum-free culture in the presence of Con A conditioned medium. This induction system may facilitate the study of lymphokine functions on antigen triggered B cells.

In T cell-replaced cultures, the antibody responses of B cells could be successfully induced when soluble SRBC membrane proteins were used as antigens. It thus indicates that antigen together with lymphokines are sufficient to drive B cells to become antibody secreting cells in the absence of T cells. The T cell-replaced system provides a more stable way for *in vitro* immunization and may be applied to monoclonal antibody production when in vivo immunization is difficult to be carried out.

Key words: *B cell maturation, lymphokines, in vitro immunization.*

INTRODUCTION

The events of B cell activation, proliferation and differentiation are complicated, and have not been completely elucidated. Most attention has been focused on T-dependent, MHC restricted antibody responses. Recently, an increasing number of reports have appeared on B cell growth and differentiation factors: non-antigen specific, non-MHC restricted soluble molecules, such as IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, and IFN-γ [1,2]. Most of these factors are secreted by Tcells, there is reason to wonder if the T-helper functions are carried out by soluble factors in thymus-dependent (TD) antigen stimulated antibody responses. There are two views on

this question. One holds that T-cell factors alone cannot replace T-cell function and restore the specific antibody responses of B cells. T-B cooperation is needed[3, 4]. The other holds that the helper function of T ceil is realized by secreting non-specific soluble factors[5]. These factors support the,proliferation and differentiation of B cells.

Previous reports have showed that T-cell factors are sufficient to replace T-helper function in SRBC-stimulated antibody responses[6.7]. However, it is not clear to what extent T-cell factors can replace T cells in 13 cell responses to conventional soluble protein antigens, especially when low dose protein antigens are used, that constitutes one of the most controversial issues in immunology[3,5].

Furthermore, in culture system for the study of lymphokines, bovine serum (BS) is usually added. The functions of serum components in B cell proliferation and differentiation are not well known, and the presence of serum may complicate the analysis of lymphokine function.

In the present study, we have reconstituted the anti-SRBC primary antibodyresponses of T-depleted spleen cells in serum-free culture;using Con A-stimulated murine spleen cell supernatant as the sources of T-cell replacing factors.With this T cell replaced culture system, we have also induced *in vitro* antibody responses of 13 cells to two different forms of SRBC antigens: soluble membrane proteins and SRBC ghosts.

MATERIALS AND METHODS

Animals

C57BL/6J and other inbred strain mice were obtained from animal house of Shanghai Institute of Cell Biology.

Antigens

SRBC, SRBC ghosts, SRBC membrane soluble proteins were used. SRBC were prepared from sheep blood samples. SRBC ghosts (membranes, MEMB) were prepared from SRBC by hypotonic hemolysing[8]. Soluble SRBC membrane proteins were extracted from SRBC ghosts with chelating agents and salt solution[9]. The method is chiefly as follows: 100 ml of SRBC ghost in 20 mOsM Tris-HCL buffer (0.6 mg protein/ml) were dialyzed against 2,000 ml of 1 mM EDTA-50 m*M* 2-mercaptoethanol for 48 hours at 4℃. The suspension was then centrifuged at 20,000 xg for 1 hour at 4℃. The pellet was suspended in 200 ml of 0.8*M* NaCl solution and stirred for 18 hours at 4℃. After the suspension was centrifuged at 13,000 xg for 1 hour at 4℃, the supernatant solution was withdrawn and the proteins in it were referred to as NaCl extracted SRBC membrane proteins (MP). The above protein samples were condensed and adjusted to 310 mOsM by dialysis and superfiltration. A modified Lowry procedure[10] was used to determine the protein concentration in SRBC ghosts and soluble SRBC membrane proteins.

B Cell Preparation

Murine spleen cells were treated with anti-Thy serum and guinea pig complement. The cell (chiefly B cells and macrophages) recovered from this procedure showed no proliferative activity to concanavalin A (Con A). The recovered anti-Thy treated spleen cells were referred to as T-depleted spleen cells.

Preparation of Medium and Conditioned Medium

1. RPMI 1640 medium

RPMI 1640 medium was prepared from powder with 20mM glutamine and $5x10^{-5}$M 2-mercaptoethanol (2-ME). 10% bovine serum (BS) was added just before use.

2. IMDM/Ham's F12 medium[Ill

Iscove's Modified Dulbecco's medium (IMDM) and Ham's FI2 medium were mixed at 1:1 ratio, and the following supplements were added: bovine insulin 5 mg/l, Human transferrin 5 mg/l, putrescine-2HCl 0.081 mg/l, progesterone 2×10^{-8} *M*, and hypoxanthine, thymidine, 2-ME, HEPES.

3. Lectin-free conditioned medium[12] and serum-free conditioned medium

Spleen cells (2×10^{7} viable cells/ml) were incubated with Con A (20 μg/ml) for 2hours at 37℃. After washing, the spleen cells were resuspended in RPMI 1640 medium (containing 10% BS) and incubated for 18 hours at 37℃. The culture supernatant was collected and sterilized through 0.45 μm filter. The medium was referred to as lectin-free conditioned medium (LF--CM).

Spleen cells (5×10^{6} viable cells/ml) were incubated with Con A (2 μg/ml) in IMDM/Ham's F12 serum-free medium for 28 hours at 37℃. The culture supernatant was collected, sterilized through 0.45 μm filter, aliquoted and stored at -30℃ or 4℃ (used within one week). The medium was referred to as serum-free conditioned medium (SF-CM). When used, α-methyl-D-mannoside was added at final concentration of 20 m*M* to inhibit the action of remnant Con A in SF-CM

Culture system for in vitro immunization

Complete spleen cells or T-depleted spleen cells were cultured at 37℃ in 96-well plates with different forms and concentrations of SRBC antigens, 10^{6} viable cells per well with a total volume of 255 μl and 8 wells as a group. After five days, antibody producing cells were measured with direct hemolytic plaque assay. The representative results were shown as mean PFCs± SD and similar results have been obtained from experiments repeated for more than two times.

Hemolytic plaque assay

Plaque forming cells (PFCs) were determined by the slide version of Jerne plaque assay with some modifications[13]. Cells collected from wells were mixed with SRBC and guinea

pig complement.The mixture was filled into a small glass chamber (O.17mm thick) and incubated at 37℃ for 45 min. Number of plaques in a designated area of glass chamber was counted. and PFCs per culture were calculated.

EESULTS

1. Induction of primary anti-SRBC antibody responses in T cell-depleted spleen cell cultures supported by LF-CM.

In preliminary experiments, 9 inbred strains of mice were compared for their PFC responses to SRBC. C57BL/6J mice responded best of all and thus were chosen as experimental animals throughout the experiments.

T cell-depleted spleen cells were cultured in medium containing 10% BS and 40% LF-CM: and 15 μl of 0.1% SRBC was added as antigen. After five-days at 37℃, the anti-SRBC PFCs were assayed. As the results shown in Tab. 1, SRBC

Tab. 1 *In vitro* induction of anti-SRBC antibody responses in T cell-replaced cultures

Treatements	PFCs/culture±SD
SRBC	0±0
SRBC+LF-CM	1071±29
SRBC+LF-CM+LPS 0.2μg/well	1682±70
SRBC+LF-CM+LPS 1μg/well	2347±134
SRBC+LF+CM+LPS 2μg/well	1698±327
SRBC+LF+CM+LPS 1μg/well	67±35
SRBC+LF-CM	144±0

Anti-Thy treated spleen cells were cultured in medium containing 10% BS and 40% of lectin(Con A)-free conditioned medium(LF-CM).

antigen alone could not induce anti-SRBC antibody responses from the T-depleted spleen cell culture, the presence of LF-CM restored the primary antibody responses of B cells to SRBC. However, in absence of antigen, either adding LF-CM alone or together with polyclonal activator LPS, only low number of PFCs could be induced.

2. Induction of anti-SRBC antibody responses in T cell-depleted spleen cells supported by SF-CM

LF-CM was prepared in medium with serum while SF-CM was prepared in medium without serum. We wondered if SF-CM can replace T-helper function just like LF-CM in the anti-SRBC antibody response of T-depleted spleen cells. Thus, T-depleted spleen were cultured in IMDM/Ham's F12 medium with 10% BS, and different concentrations of SF-CM were added. After culture with SRBC for 5days, the PFCs were assayed.

Results in Fig. 1 indicate that SF-CM prepared in serum-free medium is as effective as LF-CM prepared with serum. SF-CM could replace T-helper function in

SRBC-stimulated B cell responses and displayed distinct dose-response relationship.

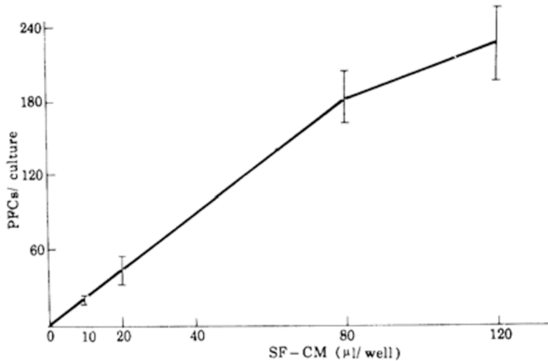

Fig. 1 Dependence of PFC responses of T cell-depleted spleen cells on dosage of SF-CM.

3. Time effect of adding SF-CM on anti-SRBC responses of T-depletJed spleen cells

T-depleted spleen cells with SRBC were cultured in IMDM/Ham's F12 meditim with 10% BS at 37℃, and 40% SF-CM was added at different time intervals after the starting of cultures. Five days later, anti-SRBC PFCs were assayed. As shown in Fig.2, the time of adding SF-CM had displayed a profound effect on the genera-

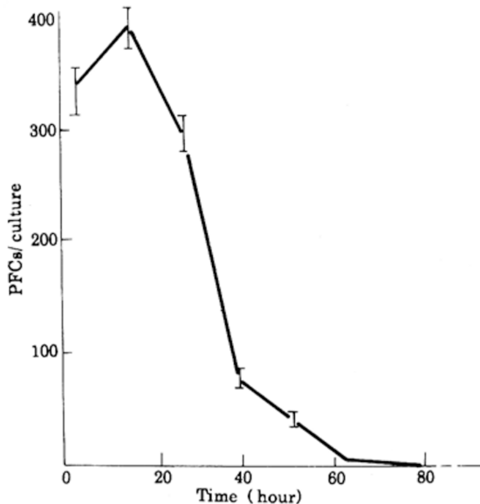

Fig. 2 Time effect of adding SF-CM on PFC responses of t Cell-depleted spleen cells.

tion ot PFCs. From 24 hours after the starting of cultures, the later the SF-CM to be added, the less PFCs to be generated, From 72-hour and thereafter, adding of SF-CM could no more restore the function of B cells to generate antibody responses.

4. Is Bovine Serum essential for the generation of anti-SBBC antibody responses in T cell-replaced culture

As shown above, T cell-depleted spleen cells could be induced to produce anti-

body forming cells in the presence of both BS and SF-CM. Then, the question raised whether BS is essential to B cells to generate antibody responses in vitro. For this, T cell-depleted spleen cell cultures with different concentrations of BS or without BS were set up. Results in Table 2 show that anti-SRBC antibody

Tab. 2 Bovine serum is not essential to the induction of anti-SRBC antibody responses in T cell-replaced cultures

Treatments		PFCs/culture±SD
Antigen + SF −CM + BS (per well)		
SRBC + BS	30 μl	0±0
SRBC+SF- CM+BS	30 μl	147±7
SRBC+SF- CM+ BS	20 μl	136±6
SRBC+SF- CM+BS	10 μl	295±0
SRBC+SF- CM+ BS	4 μl	260±56
SRBC+SF- CM+BS	2 μl	301±55
SRBC+ SF- CM+BS	1 μl	312±4
SRBC+SF- CM		317±31
− +SF-CM+BS	30 μl	8±5
− +SF - CM		0±0
− + - BS	30 μl	0±0

responses of B cell could be induced not only in serum culture, but in serum-free culture as well. This indicates that SF-CM not only could replace T-helper function, but could also support in vitro cultures.

On the contrary, as shown in Tab. 3, in complete spleen cell cultures, the cells

Tab. 3 Comparison of anti-SRBC antibody responses between complete spleen cells and T-depleted spleen cells in serum- free cultures

T-depleted spleen cells		Complete spleen cells	
Treatments	PFCs/culture±SD	Treatments	PFCs/culture±SD
SRBC	10±2	SRBC + BS	222±18
SRBC+SF −CM+ BS	567±6l	S RBC	0±0
SRBC+ SF−CM	328±6	SRBC+ SF−CM	4±4
− + SF−CM+BS	0±0	− − BS	81±2
− + SF− CM	0±0	− − −	0±0

When indicated, BS was added at 10% and SF-CM at 40% in the culture system.

were difficult to be induced to generate antibody responses in serum-free medium, whether SF-CM was added or not.

5. Induction of antibody responses of spleen cells or T-cell-depleted spleen cells to SRBC ghosts in the presence or absence of serum

Previous results were obtained with intact SRBC as antigen. If SRBC antigens

were changed into the form of SRBC ghosts, or of soluble membrane proteins, whether our culture system was still sufficient to support the generation of antibody responses induced by SRBC ghost or soluble membrane protein antigens. To this end, SRBC ghosts were prepared and used to stimulate PFC responses of lymphocytes in T cell-replaced culture.

As show in Tab. 4, the results indicate that SRBC ghosts were as immunogenic

Tab. 4 *In vitro* antibody responses of complete or T-depleted spleen cells to SRBC ghosts (MEMB)

T- depleted spleen cells		Complete spleen cells	
Antigen	PFCs/ cutlure± SD	Antigen	PFCs/ culture±SD
SRBC*	0±0		
SRBC	143±2	SRBC	272±13
MEMB 50 μg/well	151±21	MEMB 50 μg/well	24±10
MEMB 25μg/well	343±38	MEMB 25μg/well	130±20
MEMB 5μg/well	186±35	MEMB 5μg/well	105±8
MEMB 2μg/well	196±6	MEMB 2μg/well	85±10
-	18±4	-	32±0

T cell-depleted spleen cells were cultured in medium containing 10% BS and 40% SF-CM; complete spleen cells were cultured in medium containing 10% BS.
* No SF-CM was added in this group of culture.

and antigenic as SRBC; SRBC ghosts could induce PFC responses in both complete and T-depleted spleen cell cultures. Furthermore, SRBC ghosts could induce PFC responses in serum-free T-replaced cultures (Tab. 5). However, whether with or

Tab. 5 *In vitro* antibody responses of T-depleted spleen cells to SRBC ghost (MEMB) in sermu-free cultures

Treatments	PFCs/culture± SD
MEMB +BS	4±2
MEMB+ SF−CM	848±44
MEMB+ SF−CM+LPS	1165-± 176
− +SF −CM+LPS	9±1
− + SF −CM	8±0

T-depleted spleep cells were cultured in medium containing 40% SF-CM. When indicated MEMB (Ghost) antigen was added at 20 μg per well; LPS 0.5 μg per well; and BS at 10% in medium

without serum, the results again showed the importance of antigen stimulation.

6. *In Vitro antibody responses of spleen cells or T cell-depleted spleen cells to soluble membrane proteins*

Soluble SRBC membrane proteins were prepared from SRBC ghosts and were used as antigen to induce antibody responses in spleen cell cultures or T-replaced

cultures.

Tab. 6 shows that soluble SRBC membrane, proteins prepared were immunogenic

Tab. 6 *In vitro* antibody responses of complete or T-deplteed spleen cells to different forms of SRBC antigens

T- depleted spleen cells		Complete spleen cells	
Treatments	PFCs/ cutlure± SD	Treatments	PFCs/ culture±SD
SRBC	45±11		
SRBC+ SF - CM	555±0	SRBC	403±19
MEMB+ SF - CM	482±14	MEMB	305±42
MP + SF - CM	118±9	MP	151±15
– + SF - CM	23±2	-	39±1

In all cultures, 10% BS was added: and 40% SF-CM was added to T-depleted spleen cell cultures when indicated.

MEMB: SRBC ghosts, 4 μg per well

MP: SRBC membrane proteins, 2 μg per well.

They induced PFC responses not only in complete spleen cell culture, but in T-replaced cultures as well. It indicates that T cells were not indispensable in TD responses to soluble protein antigens in case T cell factors were added.

However, although three forms of antigen, intact SRBC, membrane ghose and soluble membrane proteins, could induce specific primary antibody responses, more PFCs could be induced by intact SRBC or SRBC ghost in comparison with soluble membrane proteins. The same tendency also displayed in another experiment (Fig. 3) when dose response curves of SRBC ghost and membrane proteins were compared.

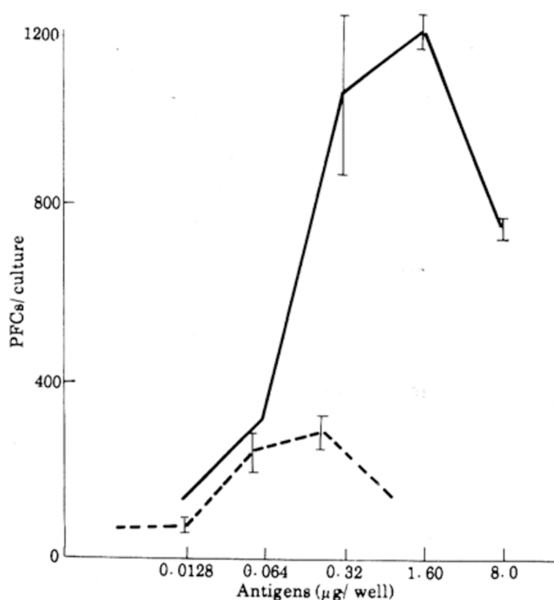

Fig. 3 Comparison of immunogenicity between SRBC ghost and SRBC membrane proteins. T-depleted spleen cells were cultured in medium containing 10 % BS and 40% SF-CM when SF-CM was not added, no PFC could be detected in the cultures.

—— SRBC ghost

······SRBC membrane proteins

DISCUSSION

1 T-helper function in antibody responses to soluble protein antigens and particulate antigens can be realized by lymphokines

T cells are necessary for TD antibody responses. In T-B cooperation, the mechanism of T cell help is not well understood. Some people pay particular attention to direct interaction of T-B cells in antibody rerponses, which is especially indispensable in soluble protein antigen induced antibody responses. The present report indicates that the direct participation of T cells was not an absolute requirement. First. in specific antibody responses to whether particulate antigens or soluble protein antigens, non-specific, non-MHC restricted lymphokines secreted by T cells can take over the function of T cells. Of course, participation of lymphokines secreted by other immune cells in presence of Con A could not be excluded. Second, the antibody responses of B cells in T-replaced cultures are not only lymphokine-dependent, but also antigen-dependent; since significant specific PFC responses can only be induced in the presence of specific antigens. Therefore, the function of antigen-specific, MHC-restricted T-B cooperation may by interpreted as that the consequence of T-B or T-M-B interactions is the releasing of nonspecific lymphokines by specific T-helper cells. Since T-B interactions are completed in very short distances, the nonspecific lymphokines released can act on specific B cells efficiently. There is evidence that in addition to macrophages, antigen specific B cells are highly efficient (in the presence of low dose antigens) tospecific T-helper cells[14]. So that T cells are activated to secret lymphokines, including B cell growth and differentiation factors, which in turn act on B cells and drive B cells to proliferate and differentiate into antibody secreting cells. In the case of T cell-replaced culture, Con A conditioned medium may serve as substitute for lymphokines from specific antigen stimulated T-B interaction, with which and in the presence of antigen, specific B cells can be driven to become antibody secreting ceils.

2. Anti-SRBC antibody responses can be induced in serum-free culture

In lymphocyte culture, serum is usually added, and the searching for a good batch of serum constitutes one of the important technical consideration in immunological research. It is not clear whether serum components in B cell response serve as nutritional or functional molecules, or both. Mosier et al [ll] once cultured purified Bcells in serum-free medium, but the experiment failed to stimulate specific PFC responses with intact SRBC as antigen. In our T cell replaced culture system, by using Con A conditioned medium, primary antibody responses could be induced in serum-free medium with intact SRBC or SRBC ghost as antigens. The results indicated that lymphokines alone were sufficient to support B cell development in defined medium, and there was no need of extra undefined serum components for maturation of B cells. The present T cell-deple.ted and factors-replacing system makes

it easier to analyze the functions of lymphokines in the development of antigen-specific B cells.

As for the soluble membrane protein antigens, we got some preliminary positive results with the same serum-free culture system; however, only small number of PFCs could be induced. It needs further study to get a clear understanding of the problems.

3. *T Cell-replaced culture may serve as an efficient and stable way to induce s'pecific antibody responses In Vitro*

The T-cell-depleted, factors-replacing and antigen specific culture system is more stable and easy to get reproducible positive results, especially when soluble SRBC membrane proteins were used as antigens, It may be due to circumvention of the problems brought forth by various T cell subsets in complete spleen cells. On the contrary, it is more difficult to control experimental conditions when complete spleen cells were used.

In T cell-replaced culture, it could ignore the balancing effects between different T cell subsets, and the T-helper function can be substituted with sufficient lymphokines in the conditioned medium. Therefore, the induction of specific antibody responses in T cell-replaced culture may serve as a practical way for In, vitro immunization of B cells, which would be especially useful in humanhybridomatechnology, and it also provides a system for the study of lymphokines function and B cell developments triggered by specific antigen.

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