Antibody Response of Mouse Splenocytes Using Mixture of Supernatants of Thymocytes, Adherent and Non-adherent Splenocytes: In-vitro Immunization-II

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We adapted one of the in-vitro immunization methods to induce antibody responses and confirmed the success of the immunization by enzyme-linked immunosorbent assay (ELISA) without hybridization. We have previously reported several methods of in-vitro immunization using different conditioned media. Here we introduce another method of in-vitro immunization using a mixture of three types of supernatant (thymocytes, and adherent and non-adherent splenocytes of mouse). Splenocytes were immunized in-vitro by a recombinant human growth hormone (rhGH) with the above conditioned media, and the results by ELISA showed a much higher optical density than the other in-vitro immunization methods that we had previously reported.

Humoral responses as a result of in-vitro immunization to soluble antigens were easily confirmed by ELISA using the above-conditioned media. This finding indicates that any other conditions thought to be critical by other researches may not be essential for in-vitro immunization.

Key Words: In-vitro immunization, ELISA, Growth hormone.

INTRODUCTION

Antibodies are one of the key elements in the immune system, and B lymphocytes are the only eukaryotic cells that can produce antibody molecules. The regulated production of antibody molecules is one of the most complex examples of eukaryotic cell differentiation. Resting B lymphocytes are activated by exogenous molecules, such as antigens and bacterial products, as well as by interleukins produced from T lymphocytes and macrophages. The activation results in the clonal expansion of antigen specific B lymphocytes and subsequent differentiation to antibody producing cells (DeFranco et al., 1987).

In our previous study (Kim et al., 1989), antibody response to recombinant human interleukin-2 from mouse splenocytes, immunized in-vitro with culture su-

pernatants of phytohemagglutinin (PHA)-stimulated adherent and non-adherent splenocytes and also of PHA-stimulated thymocytes as the conditioned media, were confirmed by enzyme linked immunosorbent assay (ELISA) without hybridization.

In this study, we repeated previous methods using another antigen, recombinant human growth hormone (rhGH), and introduced a new method of preparing conditioned media based on the theory that B lymphocytes are activated in several steps. Instead of PHA, we used a PHA-stimulated adherent splenocytes culture supernatant to stimulate non-adherent splenocytes and thymocytes.

MATERIALS AND METHODS

1. Animals

Inbred BALB/c mice were purchased from Yonsei Medical Center. Adult female BALB/c mice weighing 30-40 gm were selected and placed in individual

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metallic cages and kept under pathogen-limited conditions described by Sproll (Sproll *et al.*, 1988)

2. Antigen

Humatrope® (rhGH, Lilly, SA France) was used as the antigen for immunization at a concentration of 2 µg/ml.

3. In-vitro immunization

Spleens were surgically removed from BALB/c mice. Aseptic conditions were maintained, and the tissues were immediately placed in transport medium, Dulbecco's modified Eagle's medium (DMEM) with penicillin G 100 units/ml and streptomycin 100 µg/ml. Each spleen was gently teased apart into a single cell suspension (Hullette *et al.*, 1987). This suspension was spun for 10 minutes at 400 x g. The pellet was exposed to distilled water to lyse red cell. This was followed by two washings in phosphate buffered saline (PBS), pH 7.4 (Wybran *et al.*, 1973). Cells showing less than 95% viability tested by the trypan blue exclusion method were not used.

The washed splenocytes were resuspended in 10 ml of DMEM containing 20% fetal calf serum (FCS, Commonwealth Serum Laboratories, Melbourne, Australia) mixed with 10ml of conditioned medium containing 10 IU/ml of rIL-2; 2 µg/ml of rhGH was added, and the final splenocytes concentration was 1×10^7 cells/ml. These were incubated for five days (37°C, humidified atmosphere of 5% CO₂ and 95% (air)

4. Conditioned media

A. Preparation of PHA-activated adherent cell supernatant (ACSM-P) and PHA-activated non-adherent cell supernatant (NACSM-P)

Three ml of heat-inactivated FCS was added in a 100mm×10mm petri dish (Costar, Cambridge, USA) and incubated at 4°C for 16 hours. Ten ml of splenocyte suspension, prepared as described above, was incubated in these FCS-coated petri dishes at 37°C for 1.5 hours. Non-adherent cells were transferred to 15-ml conical tubes (Costar, Cambridge, USA) and the adherent cells attached to the petri dishes were detached physically using a rubber policeman and transferred to a 15 ml-tube.

Each cell suspension was spun for 10 min at 400 x g and resuspended in DMEM with 20% FCS to a final cell concentration of 10^7 cells/ml. Ten µg/ml of PHA was added to each cell suspension. These were incubated for two days. After centrifugation for 15 min at 400 x g, the pellets were resuspended in 10ml of

DMEM with 20% FCS and incubated for three days. Supernatants were obtained after centrifugation for 15 min at 400 x g and kept at 4°C until use.

B. PHA-stimulated thymocytes supernatant (TmSM-P)

The thymus was surgically removed from BALB/c mouse. Aseptic conditions were maintained, and the thymus was immediately placed in a transport medium, DMEM with 20% FCS and teased apart into a single cell suspension. This cell suspension was spun for 10 min at 400 x g. The pellet was exposed to mild hypotonic shock with distilled water in order to lyse red cells. This was followed by two washings in PBS, pH 7.4. Cell viability was over 90%. The washed thymocytes were added to 10ml of DMEM with 20% FCS containing 10µg/ml PHA to the final cell concentration of 10^7 cells/ml and incubated for two days. These were spun for 15 min at 400 x g, and the pellets were resuspended in 10 ml of DMEM with 20% FCS and incubated for three days. The cells were removed by centrifugation for 15 min at 400 x g and supernatants were kept at 4°C before using.

C. Preparation of thymocytes supernatant activated by ACSM-P (TmSM-AP) and non-adherent cell supernatant activated by ACSM-P (NACSM-AP)

Non-adherent splenocytes and thymocytes were prepared as described above. The non-adherent cells were spun for 10 min at 400 x g in a 15-ml conical tube and resuspended in DMEM with 20% FCS to a final cell concentration of 10^7 cells/ml containing 50% (v/v) ACSM-P. After three days incubation, these cell suspensions were spun for 15 min at 400 x g, and the supernatant (NACSM-AP) was kept at 4°C until use.

The thymocytes were suspended in 20ml of DMEM with 20% FCS to a final cell concentration of 10^7 cells/ml containing 50% (v/v) ACSM-P and incubated for three days. After centrifugation for 15 min at 400 x g, the supernatant (TmSM-AP) was kept at 4°C until use.

D. The mixture of conditioned media.

The mixture of ACSM-P, NACSM-AP, and TmSM-AP was used in a ratio of 1:1:1 in volume

Other conditioned media were prepared as described in our previous report (Kim *et al.*, 1989)

5. ELISA for anti-rhGH antibody activity

Immunized splenocyte supernatants were tested for their anti-rhGH activity by ELISA. One hundred µl of rhGH, dissolved in carbonate buffer pH 9.6 at a concentration of 4 µg/ml, were used for coating the wells of polyvinylchloride microtitration plates (Costar, Cambridge, USA). These plates were incubated for 16 hours at 37°C, and the wells were washed three times

with PBS containing 0.05% Tween-20 (PTN). The wells were charged with 100 μ l of a blocking solution, 1% bovine serum albumin in PTN (PTNB), and incubated for one hour at 37°C. The wells were washed three times with PTN and charged with 100 μ l of each supernatant of immunized splenocytes as described above and incubated for one hour at 37°C. The wells were washed three times with PTN and charged with 100 μ l of peroxidase conjugated goat anti-mouse immunoglobulins, IgG, IgA and IgM (Cappel Laboratories, Malvern, USA) at 1:1000 dilution in PTNB. O-phenyldiamine hydrochloride (Behringwerke AG, Marburg, Germany) was used as an enzyme substrate (0.4mg/ml in phosphate citrate buffer, pH 5.0). The wells were washed with PTN and charged with 100 μ l of substrate and left at room temperature for 20 min. Then 50 μ l of 2.5M H_2SO_4 was added before reading. These plates were scanned on an ELISA reader (Titertek Multiskan MC, USA). Results were expressed as the optical density at 490 nm.

RESULTS

After dividing the splenocytes into adherent cells and non-adherent cells using FCS, each cell was examined with an inverted microscope. Over 95% of the adherent cells were macrophages, while the non-adherent cells were mainly lymphocytes.

Four days after immunization, large lymphoblasts appeared in the immunized splenocytes, as described previously, and then ELISA was done for their anti-rhGH activities as described previously.

We could confirm our previous result (Kim et al., 1989) using a different antigen.

First, we tried the method of Luben and Möhler (Luben and Möhler, 1980), using a simple thymus-conditioned medium (TmCM). Antibody production of mouse splenocytes cultured with TmCM did not increase considerably as compared with the control. The antibody titer did increase, apparently due to using a PHA stimulated thymus-conditioned medium (TmSM-P) instead of TmCM (Fig. 1).

Supernatant of cultured splenocytes with PHA-stimulated splenocytes-conditioned medium (SpSM-P) showed OD than that with TmSM-P. By mixing these two conditioned media, SpSM-P and TmSM-P, the antibody titer was increased further (Fig. 2).

When we used ACSM-P and NACSM-P instead of SpSM-P mixed with TmSM-P, the antibody titer was highest (K-N method, Kim et al., 1989; Fig. 3).

In the process of T-cell activation, antigen presenting cells and cytokines release from them are needed. On

Fig. 1. Antibody production of mouse splenocytes against rhGH using a PHA stimulated thymus-conditioned medium (●-●), simple thymus-conditioned medium (■-■), without any conditioned media (▲-▲) and no antigenic challenge (▼-▼).

Fig. 2. Antibody production of mouse splenocytes against rhGH using SpSM-P + TmSM-P (○-○), TmSM-P (●-●), SpSM-P (■-■), and control (▲-▲). SpSM-P: PHA-stimulated splenocytes-conditioned medium, TmSM-P: PHA-stimulated thymus-conditioned medium.

the basis of this system, we produced NACSMAP and TmSM-AP. As we assumed, using these two conditioned media instead of NACSM-P and TmSM-P, the OD was much higher than the K-N method (modified K-N method; Fig. 4). Without diluting the supernatants, the OD values were 2.338 in modified K-N method and 1.392 in K-N method.

Among the in-vitro immunization methods tested, the modified K-N method showed the highest OD value (Fig. 5).

Fig. 3. Antibody production of mouse splenocytes against rhGH using ACSM-P+ TmSM-P (●), SpSM-P+ TmSM-P (■), and control (▲). ACSM-P: PHA-stimulated adherent cell conditioned medium, NACSM-P: PHA-stimulated non-adherent cell conditioned medium, TmSM-P: PHA-stimulated thymus-conditioned medium, SpSM-P: PHA-stimulated splenocytes-conditioned medium.

Fig. 4. Two fold dilution of supernatants of splenocytes immunized in-vitro using conditioned media. Modified K-N method (●), K-N method (■), and control (▲).

DISCUSSION

Since the early 1980s, many authors developed in-vitro immunization methods to increase immune responses. Some authors used thymocytes culture su-

Fig. 5. Antibody responses of mouse splenocytes comparing several in-vitro immunization methods. 1: modified K-N method, 2: K-N method, 3: SpSM-P+ TmSM-P, 4: TmSM-P, 5: SpSM-P, 6: TmCM, and 7: control. SpSM-P: PHA-stimulated splenocytes-conditioned medium, TmSM-P: PHA-stimulated thymus-conditioned medium, TmCM: Simple thymus-conditioned medium

pernatants as the conditioned media (Luben and Möhler, 1980, Luben *et al.*, 1982). Others added sera of human-xeno-transplant bearing mice to thymocytes culture supernatants as conditioned media (Sproll *et al.*, 1988). Another report described a method immunizing the mouse splenocytes in-vitro using nitrocellulose (Sheng *et al.*, 1987). All of the above authors suspected the success of the immunization only by the appearance of the lymphoblasts before hybridization and confirmed the immunization with hybridization to produce monoclonal antibodies.

One report described the use of fresh human serum and culture supernatant from PHA-stimulated lymphocytes as the conditioned media, and they confirmed antibody response before hybridization by plaque-forming assay or radioimmunoassay (Terashima *et al.*, 1987). Immunizing human splenocytes in-vitro using PWM and irradiate T lymphocytes as stimulatory cells has been developed by other authors, and they confirmed antibody responses by ELISA before hybridization (Hullette *et al.*, 1987).

Immune responses are the result of a high degree of cellular cooperation, along with the simultaneous effects of many humoral factors derived from immune cells and other cells.

If we simplify immunization into three processes a) antigen presentation, b) immune regulation and c) antibody production, there are two major humoral compartments, X and Y, among antigen presenting cells (APC), regulatory T lymphocytes and effector B lymphocytes (Fig. 6). Here we should decide whether immune responses are successive processes from

antigen challenge to antibody production, or if immune responses can be achieved by the simultaneous actions of many humoral factors on the immune components related to immunization. Splenocytes, which are heterogenous, are composed of many kinds of cellular immune components and fulfill the cellular conditions for immunization.

If the former description is correct, mere antigen challenging or antigen challenging with the help of a non-specific membrane stimulator (S) for the activation of APC would result in antibody production from B cells in-vitro along with the successive processes of immunization pathway (Fig. 6).

We have already confirmed that with in-vitro immunization by mere antigen-challenging or antigen-challenging with the help of a non-specific membrane

Fig. 6. The simple scheme of immunization processes from antigen presentation to antibody production (S: stimulator, X: lymphokines released from APC, Y: lymphokines released from regulatory T cell).

stimulator, PHA could not result in an antibody response (Kim et al., 1989).

From this, we repeated the in-vitro immunization of splenocytes with conditioned media containing X, Y and IL-2 which have important roles in immune responses. Using FCS, splenocytes were divided into two types, adherent cells (AC) and non-adherent cells (NAC). The AC were mostly macrophages and NAC were mainly T cells. Assuming X factors could be released from AC and Y factors from NAC by stimulation with PHA, non-specific membrane stimulator, ACSM-P(X) and NACSM-P(Y) were obtained (Fig. 7).

Classically, in-vitro immunizations were done using thymus-conditioned media only, and the researchers suggested the importance of the thymus. Here we introduced thymus-stimulating conditioned media by PHA (TmSM-P) as a third conditioned media.

In our previous report, in-vitro immunization of mouse splenocytes was done using a mixture of ACSM-P, NACSM-P and TmSM-P as the conditioned media

These seemed to fulfill all the cellular and humoral conditions for in-vitro immunization, and significant antibody responses were obtained simply by ELISA without hybridization, which were also confirmed in this study.

From this, one question arises: What was the difference between the undivided and the divided splenocytes to prepare the conditioned media? There was little difference in the processing whether the splenocytes were divided into AC and NAC or not, but there was a big difference in antibody production.

Taken all together, it may be deduced that there exists intercellular regulation among the immune com-

Fig. 7. The scheme of K-N method. ACSM-P, NACSM-P and TmSM-P were introduced.

ACSM-P: PHA-stimulated adherent cell conditioned medium, NACSM-P: PHA stimulated non-adherent cell conditioned medium, TmSM-P: PHA-stimulated thymus conditioned medium.

Fig. 8. The scheme of modified K-N method. ASCM-P, TmSM-AP, and NACSM-AP were introduced.

ASCM-P: PHA-stimulated adherent cell conditioned medium, TmSM-AP: ASCM-P-stimulated thymus conditioned medium, NACSM-AP: ASCM-P-stimulated nonadherent cell conditioned medium.

ponents, and from antigen presentation to antibody production, immune responses are achieved not only through the successive mechanism of cellular components and factors released from appropriate cells step by step, but also through the simultaneous cooperative effects of these humoral factors.

When we take the flow of the processes and the close relationship between the humoral and cellular components into consideration, it might be accurate to say that Y factors were induced by X factors (modified K-N method), not by S (PHA) (K-N method) as shown in Fig. 2. So NACSM-AP and TmSM-AP were induced by stimulating NAC and thymocytes with ASCM-P instead of PHA (Fig. 8). By using the modified K-N method we obtained a double-fold antibody response compared to the K-N method. The effects of PHA on macrophage activation have not been well known, but from this result we suspect the PHA had the effect on inducing some cytokines from macrophage, too.

The lymphokines released from NAC and thymocytes by stimulation with ASCM-P might be different from those with PHA in quantity and/or quality.

Further investigation in this field remains to be studied.

Many investigators have searched among numerous factors, cells and conditions for the critical conditions necessary for in-vitro immunization. Sometimes they tried to remove some of the conditions, factors and/or cells which they thought were not necessary or add some conditions which they thought to be crucial. They could not confirm antibody responses by ELISA, but did find the success of immunization after hybridization.

Borrebaeck *et al.*, suggested conditions for in-vitro immunization in the human system. Depletion of a lysosome-rich cell population containing large granular lymphocytes, monocytes and a subset of T suppressor cells were crucial before the cells could be immunized in-vitro. They used L-leucine methyl ester for this purpose (Borrebaeck *et al.*, 1987).

Hullette et al. suggested four crucial conditions for in-vitro immunization.

Firstly, T and B cells should be present in an approximately 1:1 ratio (Hullette et al., 1987, Cavagnaro & Osband, 1983, Chiorazzi et al., 1982). Secondly, T suppressors must be removed or depleted relative to T helpers (Hullette et al., 1987, Cavagnaro & Osband, 1983, Chiorazzi et al., 1982). Thirdly, growth factors must be available (Hullette et al., 1987, Cavagnaro & Osband, 1983). And fourthly, a polyclonal B cell activator must be present (Hullette et al., 1987, Cavagnaro & Osband, 1983, Chiorazzi et al., 1982, Strike et al., 1984).

Some authors reported that the importance of growth factors could be supplied exogenously as activated lymphocytes culture supernatant and endogenously by a mixed lymphocyte culture (Strike et al., 1984, Astaldi et al., 1982). But without satisfying these conditions, we could get remarkable antibody responses through a newly-developed method on the basis of reasonable concept, which fulfills only the third condition suggested previously by other investigators; namely, with only exogenous growth factors contained in supernatants of three conditioned media suggested above, the splenocytes were immunized in-vitro. We could confirm it simply by ELISA.

Not only did we think factors released by PHA-stimulation would be different from those released from other immune cells along the immunization pathway, but we also thought it was adequate that NAC should be activated by ACSM-P rather than by PHA if we consider the immunization pathway along the consecutive cells (macrophages, T lymphocytes and B lymphocytes).

We could establish a new method of the in-vitro immunization system.

Through in-vitro immunizations using ACSM-P, NACSM-AP and TmSM-AP, we could induce strong antibody responses. These conditioned media have their own roles, and their proper functions should be investigated.

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