A RAPID MICROASSAY FOR HEMOLYTIC ANTIBODY — ITS APPLICATION TO IMMUNOTOXICOLOGICAL STUDY —

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Abstract.....A rapid microassay for hemolytic antibody using an automatic spectrophotometer was developed. This microassay was parallel to hemolytic plaque forming cell (PFC) assay most commonly used to detect antibody formation. The former had more advantages than the latter did. The data obtained using arsenic and some metallic compounds as immunomodifiers suggested that this microassay was suitable for the screening of immunomodifiers in immunotoxicological and immunopharmacological studies which deal with a large number of samples within limited time.

Key words : Hemolytic antibody, immunopharmacology, immunotoxicology.

INTRODUCTION

A lot of materials have been introduced in our daily life and working environment. Some of them have been known as immunomodifiers (Koller, 1980 ; Dean et al., 1985 ; Descotes, 1986). Many others and newly introduced materials, however, have been unknown about their immunomodulating properties.

On the other hand, many immunological methods have been employed in immunotoxicological and immunopharmacological studies (Descotes, 1986 ; Vos, 1977 ; Dean et al., 1982). One of the most commonly used methods for antibody formation is hemolytic plaque forming cell (PFC) assay (Jerne et al., 1963). This assay, however, appears to be somewhat limited as a screening test of immunomodifiers. We need a rapid microassay to evaluate a large number of immunomodifiers within a short time. For this purpose, we developed a rapid microassay for hemolytic

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antibody produced *in vitro* using an automatic spectrophotometer.

MATERIALS AND METHODS

Animals :

Male C57BL/6N mice were purchased from Charles River Japan Inc., Atsugi, Japan. Mice were 8–20 weeks of age.

Antigen :

Sheep erythrocytes (SRBC) in Alsever's solution were obtained from Nippon Bio-Test Laboratories Inc., Tokyo, Japan, and washed twice with sterile 0.9% NaCl before use.

Preparation of spleen cells :

Spleens were aseptically removed from mice which were sacrificed by cervical dislocation. The spleen were teased apart in cold RPMI 1640 medium (GIBCO, Grand Island, NY, USA), supplemented with 100 U/ml penicillin, and 100 μ g/ml streptomycin (GIBCO), and passed through sterile stainless steel mesh. The cells were washed twice in the same medium. Viable cells were counted by trypan blue exclusion.

Culture for antibody formation :

The cells were adjusted to 2 to 10×10^6 cells/ml in RPMI 1640 medium supplemented with 10% fetal calf serum (Bockneck, Ontario, Canada), 25mM HEPES buffer, 200 mM L-glutamine (GIBCO) 100 U/ml penicillin, 100 μ g/ml streptomycin and 5×10^{-5} M dimercaptoethanol (Wako Pure Chemical Industries Ltd., Osaka, Japan).

The culture (total volume ; 1 ml per well) was incubated with 8×10^6 SRBC in tissue culture 24 well plate (Flow Laboratories Inc., Mclean, VA, USA) at 37°C in humidified atmosphere of 5% CO₂ in air for 4 to 6 days.

Assay of hemolytic antibody :

Each culture was pipetted to suspend cells. One hundred μ l of the cell suspension in each well was transferred to a micro-well plate (96-well flat bottom plate, Corning Cell Wells, NY). Fifty μ l of guinea pig complement (C, Kyokuto Pharmaceutical Industries, Ltd., Tokyo, Japan) appropriately diluted with gelatin-veronal buffer solution (GVB⁺⁺, barbital (0.003M)-barbital sodium (0.0018M)-NaCl, with Ca⁺⁺ (1.5×10^{-4} M), Mg⁺⁺ (5×10^{-4} M) and 0.1% gelatin, pH 7.4) (Williams and Chase, 1968) and 50 μ l of an appropriate concentration of SRBC suspended in GVB⁺⁺ were added and well mixed. Then the mixture was incubated at 37°C for various time.

After the incubation, the mixture in each well was suspended and the micro well plate was centrifuged at $1000 \times g$ for 5 min. From each well, 20 μ l of the supernatant was transferred to the well of another micro-well plate which had previously been filled with 180 μ l of distilled water.

Optical density (OD) of the diluted supernatant in each well was measured at

540 nm by an automatic spectrophotometer for micro-well plate (Immuno Reader NJ-2000, Nippon Inter Med Corporation, Tokyo, Japan). Mean and standard deviation were calculated.

PFC assay :

The number of direct (IgM) plaque-forming cells (PFC) was enumerated using a modification of the Jerne plaque technique (Jerne et al., 1963). Their mean and standard deviation were calculated.

Arsenic and metallic compounds :

Sodium arsenite (NaAsO_2 , E. Merk, Darmstadt, West Germany) was dissolved at a concentration of 500 $\mu\text{g/ml}$ as arsenic (As) in 0.9% NaCl. Cadmium chloride (CdCl_2 , Cd), mercuric chloride (HgCl_2 , Hg), chromium chloride (CrO_3 , Cr) and manganese chloride ($\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, Mn) (Wako Pure Chemical Industries Ltd.) were dissolved at a concentration of 5 or 50 $\mu\text{mol/ml}$ in 0.9% NaCl.

These solution were filter-sterilized and diluted with sterile 0.9% NaCl to appropriate concentrations. Twenty μl of the solution were added to the test cultures.

RESULTS

Optimal conditions in the microassay for hemolytic antibody :

We had determined the optimal conditions for this microassay. Normal spleen cells (6×10^6 cells/ml) were cultured with SRBC as antigen for antibody formation. After 5 days, several cultures were pooled and well mixed. The cell suspensions were used for the microassay. First, to determine the optimal concentration of SRBC we used fixed conditions of concentration of C (10% dilution) and time of incubation (60 min), which have been generally used in hemolytic assay. The data showed that 5% suspension of SRBC was optimal (Fig. 1).

Next, we examined whether or not 10% concentration of C was suitable for this microassay. By using fixed concentration of SRBC (5% dilution) and time of incubation (60 min) we confirmed that 10% concentration of C was optimal for this assay (Fig. 2).

We further examined on the time of incubation using fixed concentrations of SRBC (5% dilution) and C (10% dilution). We confirmed also that 60 min was optimal (Fig. 3).

Therefore subsequent experiments were performed using the three optimal conditions for the microassay as determined.

Comparison of the microassay for hemolytic antibody and PFC assay :

Various concentrations of normal spleen cells were cultured with the SRBC antigen for antibody formation. And the responses were examined after incubation for various days by both the microassay and the PFC assay. The responses obtained by both assays were almost similar (Fig. 4).

Fig. 1. Normal spleen cells (6×10^6 cells/ml) were cultured with SRBC for 5 days. Then, several cultures were pooled and used for the microassay. The assay was performed under the conditions of 10% concentration of C and incubation time of 60 min. The data represent mean $\pm$ S. D. ($n=6$) of each group.

Fig. 2. Normal spleen cells (6×10^6 cells/ml) were cultured with SRBC for 5 days. Then, several cultures were pooled and used for the microassay. The assay was performed under the conditions of 5% concentration of SRBC and incubation time of 60 min. The data represent mean $\pm$ S. D. ($n=6$) of each group.

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Fig. 3. Normal spleen cells (6×10^6 cells/ml) were cultured with SRBC for 5 days. Then, several cultures were pooled and used for the microassay. The assay was performed under the conditions of 10% concentration of C and 5% concentration of SRBC. The data represent mean $\pm$ S. D. ($n=6$) of each group.

Fig. 4. Various numbers of normal spleen cells (●, 2×10^6 cells/ml; ○, 4×10^6 cells/ml; ■, 6×10^6 cells/ml; □, 8×10^6 cells/ml; ▲, 10×10^6 cells/ml) were cultured with SRBC for 4, 5 or 6 days. Then, the response of each culture was examined simultaneously by the microassay and the PFC assay. The data represent mean $\pm$ S. D. ($n=6$) of each group.

Application of the microassay :

We have previously reported that high doses of As suppresses the PFC response of mice to SRBC, whereas it enhances the response at low doses (Yoshida et al., 1987). So, we examined whether the immunomodifying effect of As on the antibody response *in vitro* could be shown by the microassay. Various doses of As, at which doses we observed the immunomodifying effects of As, were added at the beginning of the culture for antibody formation. The antibody responses were examined simultaneously by the microassay and the PFC assay. The immunomodifying effect of As was shown both by the microassay as well as the PFC assay (Table 1).

Next, we used the microassay in practice for the screening of immunomodifiers. Various doses of Cd, Hg, Cr and Mn were added at the beginning of the culture for antibody formation. In this experiment, we added them as molecular weight to the culture in order to compare the strength of their immunomodifying effects. The immunomodifying effects of Cd, Hg, Cr and Mn were observed (Fig. 5).

Table 1. Immunomodulating effects of arsenic on *in vitro* anti-SRBC antibody response.^a

Exp.	As added (ng/ml) ^b	O.D. (540nm)	PFC/culture
I	0	0.228±0.039	1460±480
	50	<u>0.374±0.064</u> **	<u>2670±440</u> **
II	0	0.551±0.062	3880±880
	50	<u>0.697±0.033</u> **	4210±670
	100	0.485±0.126	2440±830*
	200	0.222±0.004**	40±30**
III	0	0.603±0.042	1500±550
	10	0.604±0.070	1700±370
	50	<u>0.665±0.029</u> *	<u>2900±590</u> **
	100	0.509±0.074*	1310±750
	120	0.487±0.066**	740±390**
	500	0.286±0.011**	0**

** : Significantly different from control P<0.01 (T-test).

* : Significantly different from control P<0.05 (T-test).

Underline: Significantly enhanced.

^a Normal spleen cells (Exp. I, II : 6×10^6 cells/ml, Exp. III : 3×10^6 cells/ml) were cultured with SRBC. After 5 days antibody response of each culture was examined by the microassay and the PFC assay. The data represent mean $\pm$ S.D. (n=6).

^b Various doses of sodium arsenite as arsenic were added at the beginning of the culture.

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Fig. 5. Normal spleen cells (3 or 4×10^6 cells) were cultured with SRBC for 5 days. Various doses of metallic compounds (n mol/ml) were added at the beginning of the culture. The data represent mean $\pm$ S. D. ($n=6$) of each group.
*: Significantly different from control $P < 0.05$ (T-test).

DISCUSSION

Our results indicate that the microassay for hemolytic antibody described in this report parallels PFC assay in quality. Plescia et al. also have measured spectrophotometrically the hemolytic antibody using tube method, and found a reproducible correlation between PFC and antibody production (Plescia et al., 1984). The PFC assay has been most frequently used in detecting antibody formation in mice. However the PFC assay has a few disadvantages. Viable cells harvested from animals or *in vitro* cultures must be assayed immediately in the PFC method. It is also time consuming to enumerate the number of PFC. Therefore, the PFC assay is not convenient for dealing with a large number of samples within limited time. On the other hand, the microassay covers these disadvantages of PFC assay, as the former is a rapid assay and consumes a small amount of materials. And there is no individual variations in assay manner of the former and one can use also stocked sera. In the PFC assay the cells which are producing antibody at the time of assay are enumerated as PFC, whereas in the microassay the antibody which have been already

produced and accumulated in the culture are assayed. Therefore, after the peak of antibody response, the number of PFC decreased, whereas OD in the microassay increased. We did not use the supernatant obtained directly from cell cultures for antibody formation because of unstable results. This unstability was probably due to the phenomenon that antibodies produced in the cell culture combined with the SRBC added as antigen, and the supernatant contained only the residual antibodies (data not shown).

Our applicative examination showed that the microassay could be used as a screening method. As suppressed the antibody formation at high doses, whereas it enhanced at low doses. This phenomenon, reported previously (Yoshida et al., 1987), was also shown by the microassay as well as the PFC assay. Cd, Hg, Cr and Mn suppressed the antibody formation at high doses, though their suppressive doses were different respectively. Relative order of suppressive activity was $Hg > Cr > Cd > Mn$. The results of suppression appear to be similar to that of several immunotoxicological reports studied by PFC assay and lymphocyte proliferative response *in vitro* (Shenker et al., 1977 ; Gaworski and Sharma, 1987 ; Lawrence, 1981 ; Fujimaki et al., 1982 ; Balter et al., 1982). On the other hand, Cd enhanced the antibody formation at low dose as well as As did, and this consisted with other report (Fujimaki et al., 1982).

Our results indicate that this microassay is suitable for the screening of immunomodifiers in immunotoxicological and immunopharmacological studies which deal with a large number of samples within limited time as well as for general studies for antibody formation.

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REFERENCES

- Balter, N. J., Nieder, W. S. and Gray, I. (1982) : Strain variation in cadmium-induced suppression of lymphocyte transformation in mice. *Immunopharmacol.*, **5**, 111-121.
- Dean, J. H., Luster, M. I. and Boorman, G. A. (1982) : Methods and approaches for assessing immunotoxicity : An overview. *Environ. Health Perspec.*, **43**, 27-29.
- Dean, J. H., Luster, M. I., Munson, A. E., et al. (1985) : *Immunotoxicology and Immunopharmacology*. Raven Press, New York.
- Descotes, J. (1986) : *Immunotoxicology of Drugs and Chemicals*. Elsevier, Amsterdam.
- Fujimaki, H., Murakami, M. and Kubota, K. (1982) : *In vitro* evaluation of cadmium-induced augmentation of the antibody response. *Toxicol. Appl.*

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Pharmacol., **62**, 288–293.

- Gaworski, C. L. and Sharma, R. P. (1978) : The effects of heavy metals on [³H] thymidine uptake in lymphocytes. *Toxicol. Appl. Pharmacol.*, **46**, 305–313.
- Jerne, N. K., Nordin, A. and Henry, C. (1963) : The agar plaque technique for recognizing antibody-producing cells. *Cell-Bound Antibodies* (B. Amos, and H. Koprowski, ed). p. 109–112. Wister Institute Press, Philadelphia.
- Koller, L. D. (1980) : Immunotoxicology of heavy metals. *Int. J. Immunopharmacol.*, **2**, 269–279.
- Lawrence, D. A. (1981) : Heavy metal modulation of lymphocyte activities I. *In vitro* effects of heavy metals on primary humoral immune responses. *Toxicol. Appl. Pharmacol.*, **57**, 439–451.
- Plescia, O. J., Pontieri, G. M., Brown, J., et al. (1984) : Amplification by macrophages of prostaglandin-mediated immunosuppression in mice bearing syngeneic tumors. *Prostaglandins Leukotrienes Med.*, **16**, 205–223.
- Shenker, B. J., Matarazzo, W. J., Hirsch, R. L., et al. (1977) : Trace metal modification of immunocompetence I. Effect of trace metals in the cultures on *in vitro* transformation of B lymphocytes. *Cell. Immunol.*, **34**, 19–24.
- Vos, J. G. (1977) : Immune suppression as related to toxicology. *CRC Crit. Rev. Toxicol.*, **5**, 67–101.
- Williams, C. A. and Chase, M. W. (1968) : Appendix II, Buffers. *Methods in Immunology and Immunochemistry*, Vol. II. Physical and Chemical Methods (C. A. Williams, and M. W. Chase, ed). p. 388. Academic Press Inc., London.
- Yoshida, T., Shimamura, T. and Shigeta, S. (1987) : Enhancement of the immune response *in vitro* by arsenic. *Int. J. Immunopharmac.*, **9**, 411–415.