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Studies on a Simple Method for the Preparation of Anti-Sheep Erythrocytes Rabbit Serum*

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I. INTRODUCTION

Much attempts have been made on the preparation of a potent hemolysin by the ways being in economic and laborious procedures in conducting complement fixation test.

In conventional method, rabbits were injected intravenously 4 to 10 ml. of a 50% suspension of washed sheep erythrocytes three to five times every four to five days interval. This method of immunization may bring about a serious anaphylactic shock with inducing a less hemolysin formation.

Rapp(1953) developed a new method for the preparation of hemolysin inoculating 0.1 ml. to 4 ml. of a boiled sheep erythrocytes stromata into rabbits via ear vein eleven times every day. The method may result in higher titered hemolysin formation if the procedures are properly conducted, otherwise, it may cause a relatively severe side reaction as well as a fluctuation of a individual immune response. Furthermore, the complexity of the antigen preparation and immunization procedures gives much trouble in adopting this method of immunization.

In this paper, the method of hemolysin formation using the formalin treated sheep erythrocytes were studied. And the method is new and simple for the preparation of hemolysin in economical, unlaborious and time saving procedures than any other methods which have been previously known.

II. MATERIALS AND METHODS

Rabbits: Rabbits not weighing less than 2.5 kgs. of body weight were employed in this experiment. They were divided into several groups without sexual difference, and each of which consisted with four rabbits.

Sheep Erythrocytes: Sheep blood was collected aseptically in equal volume of modified Alsever's solution (Bukantz et al. 1946). The mixture of blood and Alsever's solution was washed with 0.85% saline solution by centrifugation at 1,000 r.p.m. for 10 min. for three times. After the last centrifugation, the supernatant fluid was removed and the packed erythrocytes were made.

Antigen Preparation: Washed sheep erythrocytes were made a 10% suspension in 0.5 and 2% formalinzed saline solution(Wako made, 37% formalin). The suspension was gently mixed before it was returned to 70°C water bath, and blood was occasionally shaken during overnight incubation to give a homogeous mixing with formaline. The formalininzed erythrocytes were stored in refrigerator and used as an original antigen. Formalinized erythrocytes were washed with physiological saline by centrifugation at 1,000 r.p.m. for 10 minutes several times until the trace of hemolysis was washed out completely, and the sediment was made a 20% suspension into 0.85% NaCl solution before every injection.

On the other hands, some modifications such as a

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substitution of modified Alsever's solution to a physiological saline, and with or without wash off of formalin excess were made.

Hemolysin Preparation: All the rabbits were received antigen intravenously as scheduled in tables. On 18th day after the first injection, blood was collected by heart puncture and serum was harvested. It was heat inactivated at 56°C water bath for 30 minutes and stored at −20°C deep freezer.

Hemolysin Titration: Diluent used in the hemolysin titration was Veronal NaCl buffered solution pH 7.2 (Mayer et al. 1948). By employing a Kahn pipette, the hemolysin was mixed with diluent to give 10^{-2} concentration of the hemolysin. Sub-dilutions were made from this mixture. A 4% sheep erythrocytes were made by centrifugation at 1,000 r.p.m. for 10 minutes three times and suspended in the diluent.

Guinea pig serum was collected from 5 healthy male and pooled before making a dilution in Veronal-NaCl buffered solution. A two full units ($^1/_2$ dilutim) of guinea pig complement was used throughout the studies.

In conducting hemolysin titration, the tubes were filled with 0.1 ml. of diluted hemolysin, 0.1 ml. of 4% sheep erythrocytes, 0.2 ml. of two units of complement and 0.4 ml. of diluent. After a gentle mixing, the tubes were placed at 37°C water bath for 30 minutes and read the result. The reciprocal of the highest dilution of hemolysin, in which the sheep cells were lysed completely, was recored as a hemolysin titer. Furthermore, to avoid some erronous results, the control systems were also adopted simultaneously. Other detailed procedures are illustrated in table I.

TABLE 1 : Titration of Hemolysin

Tube No.	1	2	3	4	5	6	7	8	9	10	11	12	13
Hemolysin Dilution	1/1T※	1/2 T	1/3 T	1/4 T	1/5 T	1/6 T	1/7 T	1/8 T	1/9 T	1/10T	Controls		
Hemolysin ml.	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1(1/1T)	0	0.1
Sheep RBC, 4%, ml.	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Complement 1/25, ml.	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0	0.2	0
Dilluent ml.	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.6	0.5	0.6

37°C, 30 minutes incubation

* 1/1,000 dilution of hemolysin

III. EXPERIMENTAL RESULTS AND DISCUSSION

1. Effect of Immune Dose and Time of Inoculation on hemolysim formation:

This experiment was conducted to establish the most favorable immune schedule with comparing the results responded to varying dose of inoculum and time of injections.

The antigen was a 20% suspension of erythrocytes treated with 0.5% formalin and excess formalin was eliminated before every injection. Group A rabbits were once received with large dose of inoculum(15 ml.) on lst day, and bleeding was made on 18th day after the antigen inoculation. Group B rabbits were inoculated with a small dose of inoculum on the first day and followed a large dose on 5th day after the first injection. The rabbits in group C, D and E were injected with varying dose of small inoculum four times every four days interval. All the rabbits were bled on 18th day from the beginning of immunization and the serum was subjeted to hemolysin titration.

The results are illustrated in table 2. And the results indicate that group C was possible to form more than 3,000 hemolysin units, and group Eshowed 10.000 units of hemolysin titer.

On the other hands, an increased dose of inoculum as shown in group D reveals a moderate response on hemolysin formation. However, a large dose of inoculum in single or twice inoculation as presented in group A and B shows only a low degree of immune response.

The results indicate that formalin treated sheep erythrocytes induced the formation of a higher titer hemolysin without causing the anaphylactic shock, and a continuous antigenic stimulation with a relatively small dose of inoculum enhanced a pronounced immune response than that of single inoculuation with large dose.

TABLE 2 : Effects of immune dose and time of inoculation on hemolysin titer

Grop	Treatment	Dose of Inoculum (ml.) Day of Inoculation and Bleeding 1st	5th	9th	13th	18th	No. of Rabbits	Hemolysin titer
A	0.5%formalin 20%Sheep Cell	15.0	—*	—	—	Bleeding	1	< 1,000
							2	< 1,000
							3	< 1,000
							4	< 1,000
B	0.5% Formalin 20% Sheep Cell	0.1	10.0	—	—	Bleeding	1	< 1,000
							2	< 1,000
							3	< 1,000
							4	< 1,000
C	0.5% Formalin 20% Sheep Cell	0.5	1.0	1.5	2.0	Bleeding	1	4,000
							2	3,000
							3	4,000
							4	3,000
D	0.5% Formalin 20% Sheep Cell	0.5	1.0	3.0	4.0	Bleeding	1	8,000
							2	8,000
							3	8,000
							4	8,000
E	0.5% Formalin 20% Sheep Cell	1.0	2.0	3.0	4.0	Bleeding	1	>10,000
							2	>10,000
							3	>10,000
							4	>10,000

* Not injected.

2. Effect of Formalin Excess on hemolysin formation: It was observed that erythrocytes treated with 0.5% formalin was slowly lysed during several days storage in refrigerator, but stimulated a higher degree of immune response without lossing of antigenecity, if the cells were injected after washing off formalin excess (group C, D and E).

This test was designed to pursuit the effect of formalin excess on immunogenicity by inoculation of formalinized erythrocytes without washing off formalin excess through the immunization.

Rabbits were injected, with a small dose of inoculum, according to the immune schedule which was suggested to be favorable in previous experiment. The results are presented in table 3. Group F rabbits showed only a low degree of hemolysin formation, of which titers are less than 1,000 units. Increased dose of inoculum results in much individual fluctuation on hemolysin formation [illegible] Group G [illegible] the final inoculation, one of which was succumbed.

These undesirable results suggest that the decrease of antigen concentration owing to the partial hemolysis during the storage caused to a lesser degree of antigenic stimulation on hemolysin formation. However, group H rabbits might have shown more than 10,000 hemolysin units as shown in group E, even though onethird of the antigenic had cells had been destroyed.

On the other hands, formaline excess may bringout anaphylactic shock as well as the surpression of hemolysin formation. The effects of formalin excess on anaphylactic and immune surpression were not clarified in this experiment, and further studies were needed to explain them.

3. Effect of Formalin Concentration and Diluent on hemolysin formation: To prevent a partial hemolysis of sheep erythrocytes treated with 0.5 % formalin saline, and to avoid the laborious washing procedures of formalin excess before every injection, [illegible] In preliminary test, 2% formalin was shown to be

TABLE 3 : Effects of formalin excess on immunogenicity

Group	Treatment*	Dose of Inoculum (ml.) Day of Inoculation and Bleeding 1st	5th	9th	13th	18th	No. of Rabbits	Hemolysin titer
F	0.5% Formalin 20% Sheep Cell	0.5	1.0	1.5	2.0	Bleeding	1	<1,000
							2	<1,000
							3	<1,000
							4	<1,000
G	0.5% Formalin 20% Sheep Cell	1.0	2.0	3.0	4.0	Bleeding	1	2,000
							2	1,000
							3	<3,000
							4	4,000
H	0.5% Formalin 20% Sheep Cell	1.5	3.0	4.5	6.0	Bleeding	1**	—
							2	1,000
							3	<1,000
							4**	5,000

* After formalin treatment, a 20% cell saspension was injected without wash off formalin excess.
** Rabbits showed naphyalactic shock on final inoculation, and one of them was died.

he lowest concentration, with which the treated erythrocytes were not lysed, with or without wash off excess formalin for a relatively long poriod of storage.

The rabbits in group I were received erythrocytes which were treated with 2% formaline saline and washed off excess formalin before every injection. The antigen injected in group J rabbits was a 20% suspension of 2% formalin treated erythrocytes, which were washed off

TABLE 4 : Effects of Formalin Concentration and Diluent

Group	Treatment	Dose of Inoculum (ml.) Day of Inoculation and Bleeding 1st	5th	9th	13th	18th	No. of Rabbits	Hemolysin Titer
I	2% Formalin* 20% Cell	0.5	1.0	1.5	2.0	Bleeding	1	<1,000
							2	<1,000
							3	<1,000
							4	<1,000
J	2% Formalin** 20% Call	0.5	1.0	1.5	2.0	Bleeding	1	<1,000
							2	<1,000
							3	<1,000
							4	<1,000
K	0.5% Formalin*** 20% Cell(Als.)	1.0	2.0	3.0	4.0	Bleeding	1	<1,000
							2	<1,000
							3	<1,000
							4	<1,000
L	0.5% Formalin*** 20% Cell(Als.)	2.0	4.0	6.0	8.0	Bleeding	1	<1,000
							2	<1,000
							3	<1,000
							4	<1,000

* Formalin excess was washed before every injection
** Aftar formalin treatment of the erythrocytes, the excess formalin was washed and a 20% cell suspension was kept in refrigerator throughout the immunization.
*** After formalin treatment, the formalin excess was washed and the sediment was made a 20% cell suspension into Alsever's solution.

formalin excess and stored in refrigerator throughout the immunization.

Group K and L rabbits were received the erythrocytes suspended in modfied Alsever's solution after 0.5% formalin treatment and a wash off formalin excess. The immunizing methods and the results are illustrated in table 4.

All the rabbits susjected to this experiment showed only a low degree of immuneresponse, of which titers are less than 1,000 hemolysin units. Although the erythrocytes treated with 2% formalin saline and with or without a wash off excess formalin were not lysed in refrigerator for several days of storage, they showed only a negligiable immune response. However, the erythrocytes treated with 0.5% formalin solution were lysed slowly, they stimulated a higher degree of hemolysin formation if excess formalin was eliminated befor every injection.

A lesser stimulation of antibody formation with higher concentration of 0.5% formalin may due to the deterioration of antigenic structure of sheep erythrocytes.

In fact, 2% formalin may cause erythrocytes to tighly fixed and force a relatively firm clumps in process of centrifugation than that of 0.5% formalin. Furthermore, hemolysed cell components may act as an important role in the formation of hemolysin production in rabbits.

Substitution of Alsever's solution to saline, showed a poor immune response. This may due to the fact that Alsever's solutions blood preservatives and it may give surpressionon the antibody formation,

Throughout the studies, it was suggested that formalin treated erythrocytes enhanced hemolysin production with a relatively small dose of inoculum and in shorter time of period. In accordance with the relatively lower concentration erythrocytes, it was unable to cause hypersensitive state or other undesirable side reactions in rabbits. It is also noteworthy that the procedure is rather economic, unlaborious, time saving and better qualified in the view of no individual variations in the production of high titered hemolysin in rabbits.

IV. CONCLUSION

A potent hemolysin showing more then 10,000 hemolysin unit was easily obtainable from the immunized rabbits which were received via ear vein 10 ml. of a 20 % suspension of erythrocytes treated with 0.5% formalin saline 4 times every four days interval andbled on the fifth day after last injection of ant antigen.

V. REFERENCES

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抗緬羊赤血球 家兎血淸(溶血素) 生產에 關한 硏究

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全 允 成

Formalin 處理 緬羊赤血球를 溶血素 生產用 抗原으로 家兎의 耳靜脈에 接種하였던바 高價의 陽性血淸을 얻을수 있었음으로 報告하는 바이다.

Formalin 0.5% 食鹽水에 하룻밤동안 37°C 恒溫水槽에서 處理한 緬羊赤血球 浮遊液 1.0ml. 2.0ml. 3.0ml. 4.0ml.을 4日間隔으로 4回 家兎의 耳靜脈에 接種하였으며 最終抗原接種後 5日만에 採血하므로서 10,000單位 以上의 溶血素力價를 갖는 家兎血淸을 生產할 수 있었다.

이 方法의 特徵은 抗原의 製造나 免疫方法이 지금까지 알려져온 어떤 方法보다 더 簡便하며 家兎에 對한 過敏性 反應이 없으며 個體差異없이 溶血素를 生產하는 것이 특징이다.