Changes in immune serum of *Toxoplasma gondii* following administration of *T. gondii* lysate antigen

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Abstract

The relationship between cell-mediated immunity and changes in the serum components in *Toxoplasma*-immune cattle after injection of *Toxoplasma* lysate antigen (TLA) was investigated. The serum albumin decreased from 0 hour (approx. 40%) to 12 hours (approx. 37%) and returned by 48 hours (40%). In three of 5 cases, particular protein fraction (M. W. of approx. 12,000) were detected intensively in sera tested 6, 12 and 24 hours but less apparent in sera tested 48 hours after TLA stimulation. Two substances were detected in the culture supernatant of peripheral lymphocytes from *Toxoplasma*-immune cattle stimulating *in vitro* with TLA, but not in the control. The electrophoretic mobility of the substances was faster than albumin. A peak activity of macrophage migration inhibitory factor (MIF) in sera was showed at 3 hours after stimulation, and *Toxoplasma* growth inhibitory factor (Toxo-GIF) 12 hours after stimulation. Toxo-GIF activity showed mainly in the β-and γ-globulin fractions. MIF activity was transferred from the β-globulin fraction 3 hours to the albumin fraction 48 hours after stimulation. These results led to the conclusion that MIF and Toxo-GIF activities changed in response to TLA stimulation increasing initially. There was no relationship between these activities and the proportions among the albumin and α-, β-and γ-globulin fractions.

Key words; *Toxoplasma*, Serum components, Bovine, *Toxoplasma* lysate antigen, Lymphokines

Introduction

It was reported that the survival rate increased in mice inoculated with *Listeria monocytogenes* or *Salmonella typhimurium* when mice were previously inoculated with *Toxoplasma gondii*[13]. These infections were known to relate to cell-mediated immunity of the hosts. Serum level of lymphokines (LKs) increased in animals which were immunized

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with *T. gondii* and later injected with *Toxoplasma* lysate antigen (TLA)[11,14,21,22,23].

Mechanisms of cell-mediated immune response have also been studied *in vitro*. It has been repoted that *Toxoplasma* multiplication in normal macrophages was inhibited by the culture supernatant of *Toxoplasma* immune lymphocytes incubated with *Toxoplasma* antigen[1,3,8]. This phenomenon has also been observed in macrophages of human[2,3], murine[9,15,16,17,18] and hamster[6]. The inhibition of *Toxoplasma* multiplication is apparently caused by substances from immune lymphocytes. These phenomenon were found in *Toxoplasma* infections of many animal species. Since large amounts of serum are needed to examine for quantitative and qualitative changes of the immune serum, cattle are therefor highly suitable experimental animals. This report deals with the appearance of increased immune mediators in cattle chronically infected with *T. gondii* and later inoculated with TLA. Quantitative and qualitative canges of these mediators in the serum were examined at given intervals after inoculation of TLA by using several electrotion.

Materials and methods

Preparation of Toxoplasma Lysate Antigen

Toxoplasma lysate antigen (TLA) was prepared using the method of Jacobs and Lunde[5], modified by Igarashi et al[4].

Preparation of serum

Five Holstein bulls were inoculated intramuscularly with 2.5×10^8 *T. gondii* tachyzoites twice with 4 weeks interval. Sera were collected 4 weeks after the second inoculation and they were used as the chronic-stage *Toxoplasma*-infected sera.

The same bulls were used to examine the effect of TLA on chronically infected cattle. They were given 1,000 μg of TLA intravenously each, and their blood was obtained at 0, 3, 6, 12 and 48 hours later. The sera were fractionated individually by cellulose acetate electrophoresis and sodium dodecyl sulfate plyacrylamide gel electrophoresis (SDS-PAGE). The sera for determination of the macrophage migration inhibitory factor (MIF), and the *Toxoplasma* growth inhibitory factor (Toxo-GIF) activities, and raw-starch gelelectrophoresis were pooled.

Preparation of bovine peripheral lymphocytes

Peripheral lymphocytes and supernatants of lymphocytes of chronically *T. gondii*-infected cattle were collected by the Conray 400-Ficoll method[24]. The cells (1×10^6 cells/ml) was incubated at 37°C in a humid incubator of 5% CO_2 for 12 hours. Nonadherent cells were collected and the cell mixtures were centrifuged at 750 g and 4°C for 10 min. The sedimented cell concentrations were adjusted to 1×10^7 cells/ml and divided equally into two portions, one of which was treated with 50 μg of TLA per ml and the other was control. Then the cells were incubated at 37°C in a humidified incubator for 48 hours. For concentrating the protein content, the resulting supernatants was dialysed in 30% polyethylene glycol 6,000 (Wako Pure Chemical Industries, Lid., Osaka) using seamless cellulose tubing (Visking Co., U. K.).

Assay for macrophage migration inhibitory factor (MIF)

The agarose droplet method of Postlethwaite and Kang[12] modified by Igarashi et al.[4] was used.

Assessment of Toxoplasma growth inhibitory factor (Toxo-GIF)

Activity of Toxo-GIF was assessed using the method of Takei et al.[23]. Monolyer of bovine peripheral monocytes were inoculated with *Toxoplasma* tachyzoites. After incubation for 1 hour, monolyers were rinsed with TC-CS to remove non-adherent tachyzoites, then they were cultured with 1 ml of fresh full bovine serum (6,000~8,000 μg/ml), or serum albumin or globulin fractions (2,000 μg/ml) within TC-CS, for 48 hours. The cultured monocytes were stained with the May-Grünwald/Giemsa stain for light microscopy.

Electrophoresis

1) Cellulose acetate membrane electrophoresis.

Cellulose acetate membrane electrophoresis was

carried out using the standard method of the Japanese Electrophoresis Association[10] with minor modifications in the buffer and the staining solution. The composition of veronal buffer was as follows, 0.83g of 5,5-diethylbarbituric acid and 6.38 g of 5,5-diethylbarbiturate (Daiichi Pure Chemicals Co., Ltd., Tokyo) in DDW of 500 ml, pH 8.6, $\mu=0.07$. The staining solution contained 0.4% ponceau 3R (Kanto Chemicals Co., Inc., Tokyo), 6.0 g of trichloracetic acid (Wako Pure Chemical Industries, Ltd., Osaka) in DDW of 100 ml.

Oxoid membrane (Separax, Jookoo Co., Ltd., Tokyo) was used as an electrophoresis carrier.

2) Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Slab-type SDS-PAGE was performed using Laemmli's method[7], modified as Suzuki[20]. Samples for electrophoresis (1-2 mg protein/ml) were treated in solution containing 1% SDS (Wako Pure Chemical Industries Ltd., Osaka), 1% β-mercaptoethanol (2-mercaptoethanol, Wako Pure Chemical Industries Ltd., Osaka) and 20% glycerin in 10 ml tris buffer (pH 6.8) at 80°C for 5 min. Three percent polyacrylamide gel in 0.125 M this-HCl buffer (pH 6.8) containing 0.1% SDS was used as concentration gel and 5~20% gradient polyacrylamlde gel in 0.375M tris-HCl buffer (ph8.8) containing 0.1% SDS as separation gel. The running buffer consisted of 0.05 M tris, 0.37 M glycine and 0.1% SDS. Electrophoresis through the concentration gel was performed at 5 mA (15 V) and at 10 mA (30~60 V) for approximately 20 hours.

3) Raw-starch gel electrophoresis.

Granular starch gels were washed with distilled water (DW) for 2 days, which was changed 3~4 times daily. Then, the gels were dried and kept overnight in veronal buffer (consisting of 1.85 g of 5, 5-diethylbarbituric acid and 10.3 g of sodium 5,5-diethylbarbiturate in DDW to a final volume of 1, 000 ml, pH 8.6, $\mu=0.05$).

The starch gels in the distance proportion of 15 : 25 from the negative side (i. e. closer to the cathode) were replaced by dry gel (approx. 12×40×2 cm), and 10 ml of serum was placed on this place. Electrophoresis was performed at 24 mA (200~300 V) for 24~26 hours at room temparature. After that, the block of starch was divided into 20 sections. Each section was left overnight in 30 ml of physiological saline and then centrifuged at 250 g for 5 min to eliminate containing starch gel particles from supernatant of decanted solution. The supernatants were dialysed to concentrate. The total protain concentration was estimated spectrophotometrically (Hitachi, Perkin-Elmer UV-Vis) at 280 μm using bovine serum albumin fraction V as standard. The each fraction V as standard. The each fraction was analyzed by cellulose acetate membrane electrophoresis and immunoelectrophoresis.

4) Immunoelectrophoresis

Glass slides precoated with a thin film of 0.6% ager in DDW (Ager Noble, Difco Laboratories, Detroit, Mich., U. S. A.) were coated a second layer of gel (3ml of 1.2% agar in veronal buffer). Electrophoresis was carried out in veronal buffer at a constant voltage of 60 V for approximately 70 min. When a precipitate was produced within 48 hours after the addition of antiserum, the slides were rinsed with physiological saline for 3 days, washed with DW, and dried at 37°C (covered with wet filter paper to ensure that the grooves were filled with water). The slides were stained by the staining solution which consisted of 0.5 g of amido-black 10B, 50 ml of methanol, 10 ml of acetic acid (99%) and 40 ml of DDW, and destained overnight in a destaining solution which consisted of 50 ml of methanol, 10 ml of acetic acid and 40 ml of DDW.

Results

Changes in components of Toxoplasma-immune serum

When the sera of the bulls were examined before and 48 hours after injection with TLA using the Eiken latex hemagglutination test (Eiken Chemical, Co., Ltd., Tokyo), they showed no increase in antibody titer against *T. gondii* antigen. These titers

were stable at 1 : 256 in one bull, 1 : 512 in two and 1 : 1024 in the two animals.

1) Cellulose acetate membrane electrophoresis.

As shown in Fig. 1, the ratio of alubumin to globulin (A/G) decreased significantly to a minimum value of 0.62 ± 0.03 after 12 hours ($p < 0.05$). Then, it returned gradually to reach the pretreatment value (about 0.71) by 48 hours after TLA injection. The albumin fraction showed the lowest value ($37.2 \pm 1.3\%$) after 12 hours and returned to its original value (about 40%) 48 hours after TLA injection. The value of α-globulin fraction showed a rise (about 25%) on 12 hours and returned to reach the pretreatment value by 24 hours after TLA injection. The β-globulin fraction showed high value 3 to 6 hours and 48 hours after TLA injection. The percentage of γ-globulin showed slightly increase from 6 hours after TLA injection and it reached about 30% on 24 hours after TLA injection, then it returned the pretreatment value.

2) Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

In three of the 5 investigated cases particular protein bands were stained strongly, when the serum was tested after TLA injection (Fig. 2). This tendency was especially obvious for the sera tented 6, 12 and 24 hours after injection. In some cases the apparent band was seen at 24 and 48 hours after stimulation with TLA. The electrophoretic mobility of these protein was similar to that of cytochrome C used here as a molecular marker. Their molecular weights were entimated to be approximately 12,000.

Comparison of components between the supernatants of Toxoplasma-immune lymphocytes incubated with and without TLA

Peripheral lymphocytes of *Toxoplasma*-immune cattle were cultured with TLA. Components of the

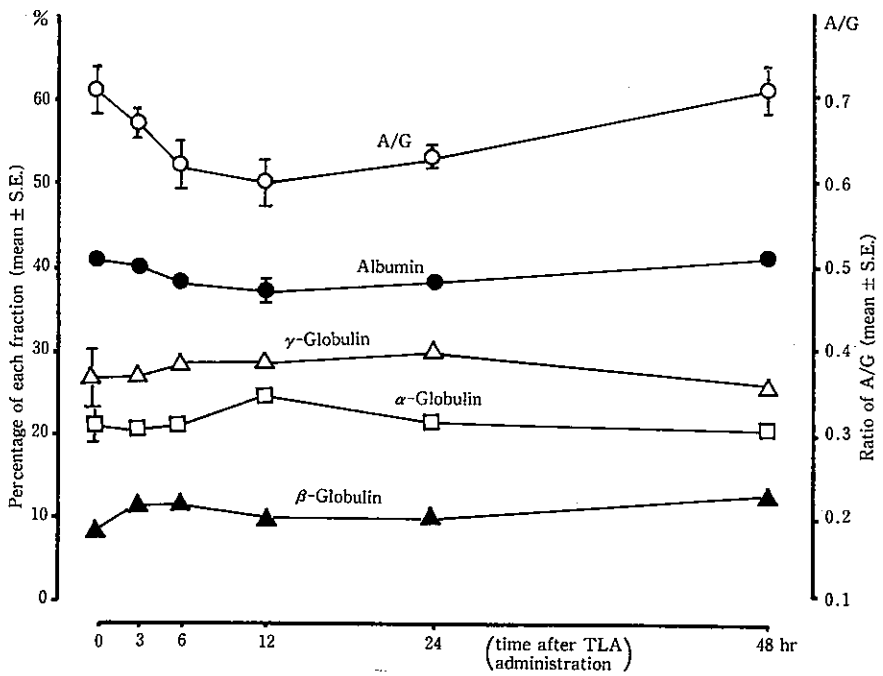

Figure 1. Quantitative changes of serum fractions in *Toxoplasma*-immune cattle at various intervals following TLA administration.

culture supernatant were separated by SDS-PAGE (Fig. 3). The culture supernatant of the TLA-treated lymphocytes contained 2 protein fractions which were not found in that of the untreated lymphocytes. Mobility of two protein bands was similar to that of cytochrome C (M. W. 12,000).

MIF and Toxo-GIF activity in Toxoplasma-immune cattle serum at different intervals after TLA administration

MIF activity (being 11% at 0 hour) in the sera increased to 58% within 3 hours following TLA stimulation *in vivo*. It dropped to a minimum of 23% in another 9 hours. Later on, it tended to increase in quantity, but its value remained below 30% (Fig. 4). Toxo-GIF activity reached to a peak 3 hours after TLA administration and decreased suddenly to −105% 6 hours after administration. It increased again to 66% by 12 hours and return to the unstimulated level of nearly 30% at 24 hours (Table 1).

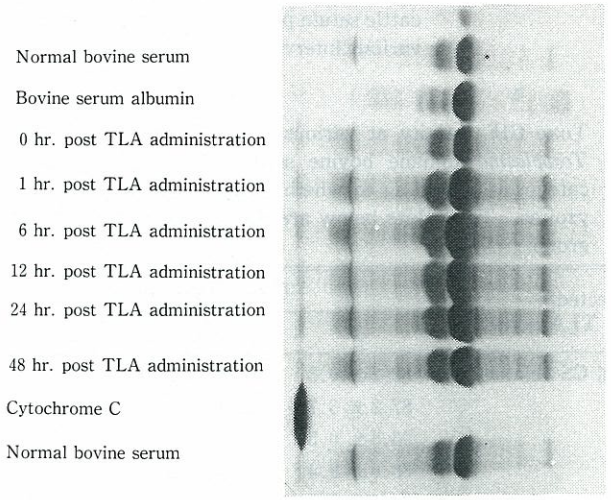

Figure 2. Quantitative changes in *Toxoplasma*-immune cattle serum at various intervals following TLA administration using SDS-PAGE.

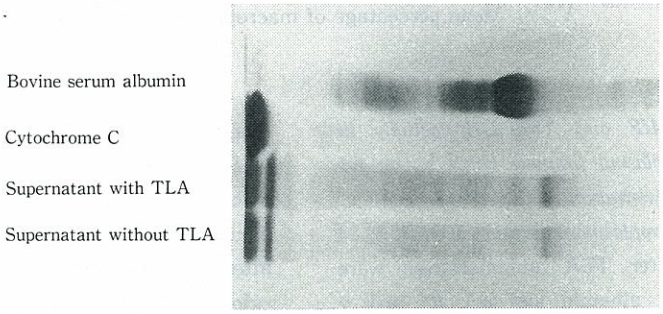

Figure 3. Comparison of components in the supernatant of *Toxoplasma*-immune lymphocytes incubated with or without TLA.

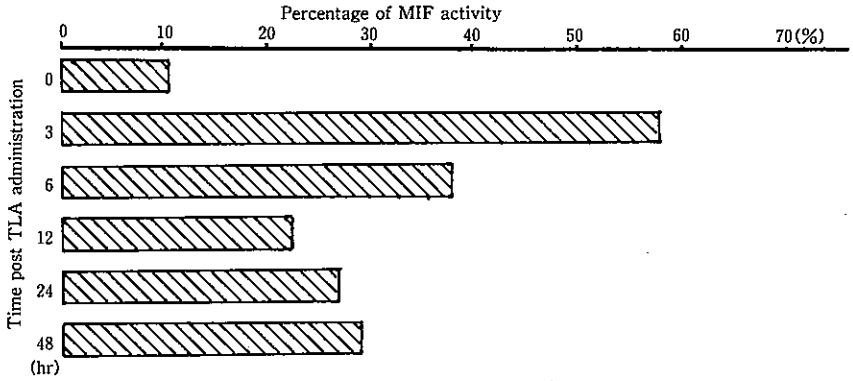

Figure 4. MIF activity of *Toxoplasma*-immune cattle serum post TLA administration at various intervals.

Table 1. Toxo-GIF activity at various intervals post TLA administration in *Toxoplasma*-immune bovine serum. Cells were grouped into three categories according to whether they contained ≧6, 1-5, or no tachyzoites. The values below are the percent of cells which belong each group.

Serum collected/ hours after TLA admin.	Tachyzoites/cell			GIF(%)*
	0	1-5	≧6	
Tc-199 20% CS (blank)	82.2±10.3	16.8±9.5	1.0±1.0	0
0 hr	87.2± 3.7	11.6±3.0	1.2±1.6	28
3 hr	93.6± 0.5	5.4±0.5	1.0±0.0	64
6 hr	63.6± 9.4	34.2±8.2	2.2±2.5	−105
12 hr	94.0± 2.7	5.6±2.6	0.4±0.5	67
24 hr	88.0± 2.3	11.0±2.7	1.0±1.2	33
48 hr	88.4± 2.3	10.8±2.3	0.8±1.3	35
Normal serum	Most of cells were destroyed.			

$$*\text{GIF}(\%) = 100 \times \left(1 - \frac{\text{Mean percentage of macrophages with } \textit{Toxoplasma} \text{ in sample}}{\text{Mean percentage of macrophages with } \textit{Toxoplasma} \text{ in blank}}\right)$$

Distribution of MIF and Toxo-GIF activity in fractions of Toxoplasma-immune cattle serum following TLA administration

The sera of *Toxoplasma*-immune cattle 3, 6, 24 and 48 hours after TLA administration were pooled, and their albumin and α-, β- and γ-globulin fractions were separated by raw-starch gel electrophoresis. Table 2 shows the MIF activity of the full serum and the globulin fractions examined 3, 6 and 48 hours after TLA treatment. MIF and Toxo-GIF activities were found in all fractions, but they varyed among the components. Thus, MIF activities of the whole serum 3 hours after treatment, was showed 58%, but the β-globulin fraction was 61%. The MIF activities of albumin and globulins fractions 6 and 48 hours after treatment, were 68 and 97%, but both of the full serum samples showed not remarkable MIF activ-

Table 2. MIF activity of each serum fraction at 3, 6 and 48 hour following TLA administration in *Toxoplasma*-immune cattle serum. All value is the percentage of MIF activity.

Time	Albumin	Fraction			Whole serum
		α-Globulin	β-Globulin	γ-Globulin	
3 hr	39	37(Alb)*	61	44	58
6 hr	93	97	79	93	38
48 hr	83	68($\beta-$)*	70	75($\beta-$)*	29

* These fractions are including ( ).

Table 3. Toxo-GIF activity at 3 and 24 hr after TLA administration are demonstrated. Cells were grouped into three categories according to whether they contained ≧6, 1-5 or no tachyzoites. The value is the percent of cells which belong to each group.

Serum fraction	Tachyzoites/cell			GIF(%)*
	0	1-5	≧6	
Tc-199 20% CS (blank)	74.6± 5.5	24.0± 4.3	1.4±1.5	0
3 hr Albumin	72.8± 8.2	26.0± 7.9	1.2±1.3	− 7
α-globulin(Alb)	------ Most of cells were destroyed.------			
β-globulin	90.0± 3.7	9.4± 3.4	0.6±0.9	61
γ-globulin	78.6±12.3	19.6±10.9	1.8±1.9	16
24 hr Albumin	71.6±10.1	25.0± 7.3	3.6±3.6	−12
α-globulin(β-)	------ Most of cells were destroyed.------			
β-globulin(γ-)	95.6± 1.5	3.8± 1.3	0.6±0.5	83
γ-globulin	------ Most of cells were destroyed.------			

$$*\text{GIF}(\%) = 100 \times \left(1 - \frac{\text{Mean percentage of macrophages with } \textit{Toxoplasma} \text{ in sample}}{\text{Mean percentage of macrophages with } \textit{Toxoplasma} \text{ in blank}}\right)$$

ities. Table 3 demonstrates for Toxo-GIF activities 3 and 24 hours after TLA stimulation. The remarkable Toxo-GIF activities were found in both of the β-globulin fractions 3 and 24 hours after treatment.

Discussion

It has been reported that serum of infected human[2,3] and mice[15,16,17] with *Toxoplasma gondii* had a factor which suppresses *Toxoplasma* multiplication. Among the factors related to cell-mediated immunity, macrophage migration inhibitory factor (MIF) and *Toxoplasma* growth inhibitory factor (Toxo-GIF) may play some roles of host diffence mechanisms in *Toxoplasma* infection.

In this study, the decrease of albumin fraction and the increase of globulin fractions were seen in the *Toxoplasma*-immune cattle serum during 24 hours after stimulation with *Toxoplasma* lysate antigen (TLA).

To our knowledge, there are no reports on the distribution of MIF activity in bovine serum fractions. If there is a species variation, the results of the present studies suggest that MIF detected 3

hours after stimulation was different in physicochemical properties from that detected after 48 hours.

Klinkert and Sorg[24] proposed that MIF might be composed of indipendent subunits and they determined molecules with MIF activity to have an M. W. of 30,000, 45,000 or 60,000. However, taking into consideration the molecular weight determined by different workers, an alternative explanation is required. MIF activity may be related to a subunit, which binds to various heterologous proteins or other substances present in the serum or medium. These findings might support to the hypothesis that MIF activity may be a property of a subunit, which may be attached to various other serum/medium components causing a change in molecular weight and electrophoretic mobility.

Youngner and Salvin[25] showed that the properties of MIF were very similar to those of immune IFN (IFN-γ). According to Shirahata and Shimizu[19], Toxo-GIF from splenic cells of immune murine share physicochemical properties with IFN. Takei et al.[23] found the maximum activity of Toxo-GIF in the plasma of *Toxoplasma*-immune beagles 24 hours after intravenous injection with TLA. It has been speculated that Toxo-GIF released by *Toxoplasma*-immune T-lymphocytes[18] may not be part of the immunoglobulin or albumin fractions of the serum[19]; using ager zone electrophoresis they found Toxo-GIF activity in the post-albumin position.

In the present studies, however, there was an increase in the α-globulin fraction at the time of the peak of Toxo-GIF activity (12 hours after stimulation), and raw-starch gel electrophoresis revealed that the activity is mainly related to the β - and γ-globulin fractions. The results of these studies led to the conclusion that these factors may change in properties with the lapse of time after TLA administration. Further investigations are required to clarify the relationships of MIF, Toxo-GIF and IFN-γ.

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トキソプラズマ抗原投与による トキソプラズマ感染血清蛋白の推移

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摘 要

トキソプラズマ慢性感染牛にトキソプラズマ抗原(TLA)を投与した後の血清成分の質的及び量的変化の推移を経時的に検討した。セルロース・アセテート膜電気泳動法から,TLA 投与後48時間以内にグロブリン画分の増加とアルブミンの減少が認められた。SDS-ポリアクリルアミドゲル電気泳動(SDS-PAGE)により,TLA 投与6,12及び24時間後の血清中に,他の時間や健常牛の血清には認められない,分子量1万2千の物質が観察された。感染牛末梢血リンパ球を TLA 存在下で培養し,培養上清を SDS-PAGE にて検討したところ,TLA 添加培養上清中に,分子量1万2千の特異的物質が観察された。マクロファージ遊走阻止因子(MIF)とトキソプラズマ増殖阻止因子(Toxo-GIF)活性については,MIF は,3及び48時間後に活性の増加を示し,Toxo-GIF は,3及び12時間後に高い活性を示した。これらの血清を生澱粉電気泳動法で4分画(アルブミン,α-,β-,γ-グロブリン)に分離し,それぞれの活性の分布を検討したところ,MIF 活性は,TLA 投与後3時間の血清では β-グロブリン分画に,48時間ではアルブミン画分に高い活性を認めた。一方,Toxo-GIF 活性は,β-,γ-グロブリン画分に認められた。