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Toxoplasmacidal Activity of Hydrolyzed Plasma from *Toxoplasma*-Immune Beagle in Heterologous Cells

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Introduction

When *Toxoplasma* lysate antigen (TLA) was injected intravenously into *Toxoplasma* (Tp) hyperimmune beagles 2 weeks after Tp rechallenge, plasma collected from the beagles 24 hr after injection inhibited the multiplication of Tp markedly in homologous cells. No changes were found in the microbicidal activity when the plasma was absorbed with anti-canine IgG (Takei *et al.*, 1981). These results indicated that an inhibitory substance contained in the plasma was not derived from antibodies specific to Tp, but was a so-called Tp growth inhibitory factor (Toxo-GIF) (Shirahata *et al.*, 1977) or a resembling factor being released from T-lymphocytes. Neither difference nor similarity is yet clarified between Toxo-GIF and gamma interferon although both of which are found in the same plasma.

An inhibitory factor or substance of Tp multiplication in homologous cell monolayers is ascertainable in the culture supernatant, or lymphokines (LKs), when spleen cells from Tp immune animals are incubated with TLA or nonspecific mitogens (Hoff and Frenkel, 1974; McLeod and Remington, 1977; Sethi *et al.*, 1975; Shirahata *et al.*, 1975, 1977; Igarashi *et al.*, 1979; Matsumoto *et al.*, 1981; Nagasawa *et al.*, 1980). Toxo-GIF (molecular weight, 30,000 to 40,000) in LKs inhibits Tp multiplication in homologous cells but not in heterologous cells (Matsumoto *et al.*, 1981; Nagasawa *et al.*, 1980.) Recently, antiviral activities not only against homologous but also against heterologous cells were demonstrated in interferon in the circulation of calves (Gresser *et al.*, 1974; Babiuk and Rouse, 1976, 1977).

Taking these results and phenomena into account an attempt was made to determine whether immune plasma, which had been chemically reduced to its low-molecular components by using enzyme reaction and hydrolysis, would inhibit Tp multiplication not only in homologous but also in heterologous cells. The same hydrolyzed plasma samples were also tested for their interferon activity.

Materials and Methods

Beagles and Tp strains used: Ninety-

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day-old parasite-free beagles of the same litter from a colony maintained in a closed environment were used. To maintain constant conditions for breeding and feeding, each of 6 dogs was placed in a separate cage and fed a limited volume of standard dog diet daily. They were allowed to have tap water *ad libitum* during the experiment. Five dogs were given about 1,400 Tp cysts of the S-273 (swine origin) strain orally. The remaining one was used as an uninfected control. The dogs were challenged intraperitoneally with 2×10^7 tachyzoites of the RH strain 50 days after the primary inoculation. They were rechallenged by another intraperitoneal inoculation with the same number of tachyzoites of the RH strain 300 days after the primary inoculation and thereafter referred to as hyperimmune beagles.

Toxoplasma antibody measurement: Antibody titers against Tp were determined by the modified method of Sabin-Feldman dye test (Kobayashi *et al.*, 1968), the Eiken latex agglutination test and the indirect immunofluorescent antibody technique. The last technique was carried out with fluorescein-conjugated rabbit anti-canine IgM and IgG prepared routinely in the authors' department.

Preparation of Toxoplasma lysate antigen (TLA): TLA was prepared by the method of Jacobs (1957). Tp tachyzoites of the RH strain obtained from the peritoneal cavities of mice 2 days after infection were washed with Hanks' balanced salt solution (HBSS). After washing, 10-fold sterile redistilled water was added. The suspension was sonicated by ultrasonic vibrator (100 w Kubota insonator, Model 200, Tokyo) for 5 min and then kept at 4 C for 24 hr. The lysate antigen was centrifuged at 10,000 rpm for 1 hr. An equal volume of 1.7% NaCl was added to the supernatant. The total protein content was estimated by the Lowry method (1951) with bovine serum albumin fraction V as a standard.

Monocyte monolayer preparation: A monocyte-rich suspension was obtained by means of a slight modification of the Conray-Ficoll method of Tsuji (1971). Ten ml of heparinized blood from the cephalic vein of healthy beagles was diluted 3 times with physiological saline solution. Five ml of the diluted blood was overlaid onto 2 ml Conray-Ficoll (10 vol. 33.8% Conray plus 24 vol. 9% Ficoll) and centrifuged at 1,350 rpm at room temperature for 20 min. Mixed lymphocyte and monocyte population contained in the upper layer of the Conray-Ficoll medium was collected and washed 3 times with HBSS and once with Tc-199 medium containing 40% heat-inactivated calf serum (Medium + 40% CS). Resuspension was made in the same medium and the concentration of the cells was adjusted to 5×10^6 cells per ml. To obtain an unalloyed monocyte preparation, 1 ml of the cell suspension was allowed to adhere to a cover slip of 15 mm in diameter contained in a plastic culture multidish tray (Limbro Chemical Co., Inc.) at 37 C for 5 days in a CO_2 incubator. One third of the medium was changed on the 1st, 2nd and 5th days and every third day thereafter to remove nonadherent cells by gentle washing with prewarmed (37 C) medium + 40% CS. More than 98% of the adherent cells were monocytes including 1 to 2% neutrophils.

Interferon assay: Interferon was assayed by the plaque reduction method using a continuous cell line of mouse L cells (clone 929), canine kidney cells (MDCK) and vesicular stomatitis virus (VSV) as the challenge virus (Taguchi *et al.*, 1976). Three ml of the samples obtained by twofold serial dilutions in minimal essential medium containing 4% CS (MEM + 4% CS) were added to the canine kidney cell and mouse L cell monolayers formed in 60 mm petri dishes and incubated at 37 C for 18~24 hr in a CO_2 incubator. The monolayers formed were washed twice with the same medium and were challenged with 100~200 plaque-

forming units of VSV for 60 min. Then, MEM+2% CS and 1% agar were overlaid in each dish. The plates were reincubated for 40~48 hr. The second overlay was performed with MEM+4% CS+0.8% agar and neutral red (1 : 20,000) and plaque were counted 4 hr later.

The interferon titer was determined by plotting per cent inhibition against different dilutions on a sheet of probit paper. It was expressed as the reciprocal of the dilution of the sample which had reduced the plaque count to 50% of the control plaque count. A standard reference interferon sample was included in each assay. The titer varied within a twofold range. One unit of interferon in this assay was equivalent to six units of a reference mouse interferon standard (G-002-904-511) provided by the Department of Health, Education and Welfare, National Institute of Health, Bethesda, USA.

Preparation of hydrolyzed plasma lymphokines (HP-LKs) from Toxoplasma immune beagle plasma: To 100 ml of plasma, 0.1 g of proteinase (refined pronase, Sigma Chemical Co.) was added. The mixture was incubated at 37 C for 12 hr to destroy the peptide bonds nonspecifically. It was then mixed with 10 ml of 10 N NaOH by constant stirring and boiled at 95~100 C for 1 hr to inactivate pronase. After cooling to 4 C, the pH was adjusted to 7.0±0.1 with 10 N HCl. The hydrolyzed plasma was filtered through a piece of filter paper. The filtrate was centrifuged at 10,000 rpm for 20 min and the supernatant was collected. As eluent, a portion of hydrolyzed immune plasma was fractionated by Sephacryl S-200 gel chromatography with 0.01 M PBS, pH 7.2 at a flow rate of 18.6 ml/hr by using a 5 AU-JEOL all automatic liquid chromatograph apparatus (Nihon Denshi Co., Tokyo). The fraction of about 7,000 of molecular weight (mw) was collected as a eluate (A), and fractionated again by Toyo pearl (Fraktgel TSK-Merck) HW-40 gel chromatography in the same manner as mentioned above to collect a fraction of about 3,000 to 5,000 of mw.

Ultraviolet (UV) and refractory index (RI) absorbance were determined by Shodex OH-PAK-B-803 microcolumn chromatography with 0.01 M NaCl at a flow rate of 1 ml/min by using a high speed liquid chromatography, including an all microcomputer mechanic (Hitachi 638-30 Type, Hitachi Works, Tokyo). The eluates obtained were pooled, dechlorinized with redistilled water by Sephadex G-15 gel filtration, and freeze-dried for storage until use. This product was called hydrolyzed plasma lymphokines (HP-LKs) for convenience. Normal beagle plasma was treated in a similar fashion and served as a control.

Other estimations of HP-LKs: For the analyses of components and properties of HP-LKs, an amino acid analyzer (Hitachi 835-50 Type, Hitachi Works, Tokyo), a dual wavelength spectrophotometer (Hitachi 557 Type) for the ultraviolet absorbance curve estimation, an infrared spectrophotometer (Hitachi 260-50 Type) for infrared absorbance spectra by the KBr tablet method, and a fluorescent spectrophotometer (Hitachi 650-60 Type) were applied.

Molecular weight determination of HP-LKs: To determine the approximate mw of HP-LKs, the column was calibrated with blue dextran (mw, 1,000,000), bovine albumin (mw, 67,000), egg albumin (mw, 45,000), alpha chymotrypsinogen (mw, 24,500), lysozyme (mw, 14,300), insulin (mw, 6,000) and insulin-chain B (mw, 3,400) by using the above-mentioned Sephacryl S-200, Toyo pearl HW-40 gel filtration and Shodex OH-PAK-B-803 column filtration.

Assessment of cell microbicidal activity: The above normal cell monolayers were infected with about 1×10^5 tachyzoites of the RH strain per dish 1 hr before the addition of either plasma or HP-LKs to each chamber. The fate of the intracellular parasites was monitored by phase contrast

microscopy of the infected cell cultures at regular intervals. Tp could be readily identified by its morphological appearance within cytoplasmic vacuoles. Thus, the cultures were stained with May-Grünwald-Giemsa stain and examined by light microscopy. The infection rate of cells was calculated from the number of parasites in vacuoles of 1,000 individual cells on each cover slip. The rate was 1 to 5 tachyzoites in one group and more than 6 tachyzoites in the other. To obtain a mean infection rate, experiments were repeated at least 3 to 5 times.

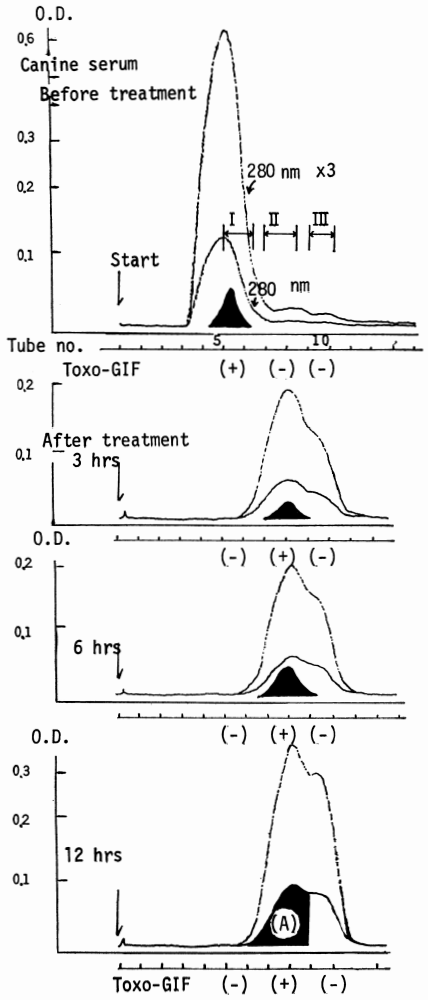

Fig. 1 Chromatography pattern on Sephacryl S-200 gel filtration of canine Toxoplasma immune serum and its enzyme-hydrolysis substance 3, 6 and 12 hours after treatment.

Note: A constant flow of 18.6 ml per hour (0.01 M PBS, pH 7.2; sample size, 0.8 ml of 50% solution) through the column (10× 700 mm) was achieved by an autoapparatus of liquid chromatograph, JLC-5 AUH type, JEOL, Tokyo.

Results

Gel filtration of pronase-treated and hydrolyzed plasmas performed at different time intervals after treatment: The results of fractionation of pronase-treated plasma by Sephacryl S-200 gel filtration are shown in Fig. 1. All the eluents obtained were fractionated into 20 parts of 4 ml each. As shown in Fig. 1 (top), untreated plasma was fractionated into 15 parts or separated into 3 peaks. Fractions I, II and III were collected. Toxo-GIF was contained in Fraction I, but not in Fractions II and III. When treated with pronase at different time intervals from 3 through 24 hr by the preparation method of HP-LKs from the plasma, it shifted to Fraction II, as marked black in the figure. At 12 hr, Toxo-GIF in Fraction II was of higher level than that at any other time of incubation and col-

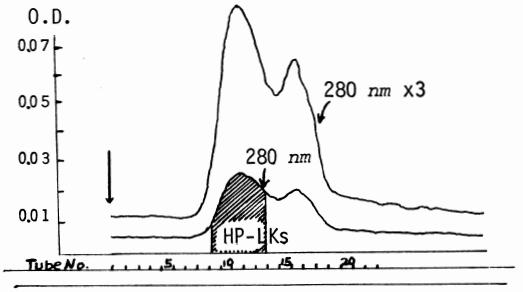

Fig. 2 Chromatography pattern of crude HP-LKs previously obtained from Sephacryl S-200 gel filtration on Toyo pearl Frakt gel 40.

Note: A constant flow of 30 ml per hour (0.01 M PBS, pH 7.2; sample size, 3 ml of 10% solution) through the column (20×700 mm) was achieved by the JLC-5 AUH liquid chromatograph.

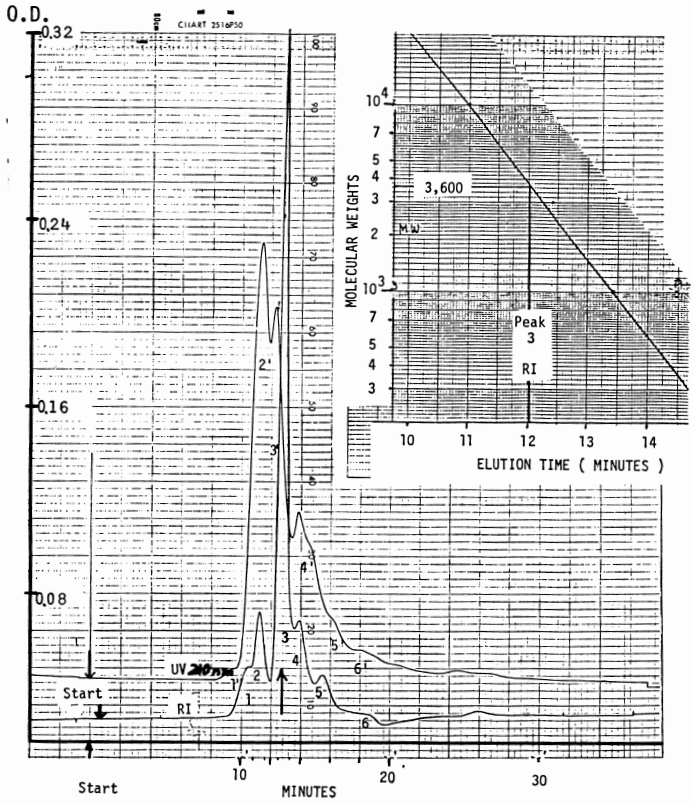

Fig. 3 Calculation pattern on Shodex OH-PAK-B-803 gel column chromatography with ultraviolet (UV) and refractory index (RI) absorbance methods.

Note: A constant flow of 1.0 ml per minutes (0.01 M NaCl; sampe size, 75 μl of 0.4% solution) through the superfine Shodex OH-PAK-B-803 packed column was achieved by the Hitachi 638-30 liquid chromatograph.

lected as eluate (A). Eluate (A) was fractionated again by Toyo pearl HW-40 gel chromatography and separated into 2 peaks, as shown in Fig. 2. Toxo-GIF was contained in the 1st peak, but not in the other peak. The eluates from the 1st peak, being called HP-LKs, were pooled and dechlorinized with redistilled water by Sephadex G-15 gel filtration. The remaining eluates were freeze-dried for storage until use.

Molecular weight determination of HP-LKs: The molecular weight of HP-LKs was estimated to be between 3,000 and 5,000 by Toyo pearl HW-40 gel chromatography. Results obtained by Shodex OH-PAK-B-803 gel column chromatography using the UV and RI methods are shown in Fig. 3. HP-LKs were present in 5 absorbant peaks demonstrated by the RI method being most prominent in the 3rd peak. Their calculated molecular weight was about 3,600. The 3rd peak obtained by the RI method was superimposed on the 3rd peak obtained by the UV method owing to the difference in time interval between the addition of the samples.

Ultraviolet and infrared absorbance spectra of HP-LKs: An infrared absorbance spectrum was drawn by the KBr tablet method. Strong absorbances were shown at 3,600~2,900 and 1,700~1,500, and middle-grade absorbances at 1,400, 1,150~1,000 and

Table 1 Toxoplasmacidal effects of *Toxoplasma* immune beagle plasma in normal beagle monocyte monolayers

Sample added in Tc-199+40% CS	*Mean percentage of monocytes with *Toxoplasma* (Tp) 48 hours after inoculation		
	0 Tp	1-5 Tp	≥6 Tp/cell
Normal beagle plasma			
10%	29.0± 8.0	31.4± 7.6	39.6±10.7
Tp immune beagle plasma			
10% TLA†	60.2±12.3	21.6± 6.5	18.2± 7.7
10% Con A†	73.6± 7.2	16.4± 5.2	10.0± 5.8
10% PHA†	72.8± 9.7	17.0± 5.2	10.2± 5.1
10% LPS†	53.6±12.0	29.4± 7.8	17.0± 6.4

*: Mean percentage±S.D. was calculated from the results of five independent results.

†: TLA, *Toxoplasma* lysate antigen; Con A, concanavalin A; PHA, phytohemaggulutinin; or LPS, lipopolysaccharide was injected intravenously 10 μg/kg of body weight, and plasma was collected from dogs 24 hours after injection.

550 wave numbers (cm^{-1}). Wave numbers 3,600 to 2,900 were indicated by the absorbance of a new -OH (hydrogen bonds) at different grades. The absorbance at wave numbers 1,700 to 1,400 was thought to be caused by -CO·NH- which was a part of polypeptides or proteins. Absorbances at wave numbers 1,150 to 1,000 might be caused by a -C-O-C- bond of carbohydrates. An ultraviolet absorbance spectrum curve was drawn by using a sample of 0.1% solution measured by a dual wavelength spectrophotometer. The maximum absorbance was estimated to be 274~276 nm.

Determination of the in vitro inhibitory effect of plasma obtained 24 hr after intravenous injection of TLA, Con A, PHA or LPS: Tp immune beagles were injected TLA, concanavalin A (Con A), phytohemagglutinin (PHA) or lipopolysaccharide (LPS) intravenously. The inoculum was always 10 μg/kg of body weight. Then plasma was separated from the blood collected 24 hr after injection. As shown in Table 1, the plasma obtained from the TLA-injected beagle showed a significant in vitro inhibition of intracellular Tp multiplication in canine monocytes, and 21.6 and 18.2% of the cells contained 1~5 and more than 6 Tp, respectively. Plasma samples obtained from the PHA- and Con A-injected beagles also inhibited Tp multiplication in the cells and 17.0 and 16.4% of them contained 1~5 Tp and 10.2 and 10.0% contained more than 6 Tp, respectively. A comparative multiplication of the parasite was observed in monolayers which had been incubated with a plasma sample obtained from the LPS-injected beagle, as well as from the normal one.

Determination of the in vitro inhibitory effect of different concentrations of HP-LKs on Tp multiplication: Tp immune plasma diluted to 66% in culture medium + 40% CS inhibited Tp multiplication in normal beagle monocytes after incubation for 48 hr, as shown in Table 2. The Sabin-Feldman dye test titers of the plasma exceeded 1 : 1,024, and IgG antibody titers greater than 1 : 4,000 were obtained by the IFA technique. This immune plasma showed a titer of 1 : 32 of interferon activity. When it was dialyzed against glycine-hydrochloric acid buffer, pH 2, at 4 C for 24 hr, the interferon activity assay revealed a drop in activity from 32 to less than 8 but no changes in interferon activity were observed when the plasma was incubated at 56 C for 60 min. This interferon was therefore regarded as gamma interferon. These results support the concept of previous authors (Youngner and Salvin, 1973;

Table 2 Toxoplasmacidal activity of *Toxoplasma* immune beagle plasma and HP-LKs in normal beagle monocyte monolayers

Sample added in Tc-199+40% CS	*Mean percentage of monocytes with *Toxoplasma* (Tp) 48 hours after inoculation			Interferon activity titer		
				Before treatment	After treating at	
	0 Tp	1-5 Tp	≥6 Tp/cell		pH 2	56 C
Normal beagle plasma						
66%	37.0±9.3	24.6±3.0	38.4±9.8	<1 : 8		
Tp chronic beagle plasma						
66%	69.6±9.6	18.4±5.9	12.0±4.5			
Tp chronic beagle plasma 2 weeks post rechallenge (hyperimmune plasma)						
66%	81.2±6.4	11.2±3.5	7.6±4.3			
Tp immune beagle plasma†						
66%	91.2±1.3	7.0±1.8	1.8±1.5	1 : 32	<1 : 8	1 : 32
Hydrolyzed plasma LKs (HP-LKs)						
0.25%	88.8±4.4	10.8±3.9	0.4±0.5	<1 : 8		
0.5 %	92.4±7.1	7.4±6.7	0.2±0.4	<1 : 8		
0.75%	96.6±2.5	3.0±2.0	0.4±0.5	<1 : 8		
1.0 %	99.1±1.4	0.9±1.4	0	<1 : 8		
2.0 %	Destroyed the greater part of monocytes (Cytotoxicity)					

*: Mean percentage was calculated from the results of five independent experiments.

†: Plasma was collected 24 hours after intravenous injection of TLA (10 μg/kg) into hyperimmune beagles.

Table 3 Toxoplasmacidal activity of hydrolyzed *Toxoplasma* immune beagle plasma (HP-LKs) in heterologous cells

Cell lines	Concentration of HP-LKs in medium†		*Mean percentage of heterologous cells with *Toxoplasma* (Tp) 48 hours after inoculation		
	Added (%)	Cytotoxicity	0 Tp	1-5 Tp	≥6 Tp/cell
Bovine monocytes	0	0	29.7±13.7	41.5± 2.3	28.8±10.8
	0.75	1.0	82.4±13.1	13.1± 8.9	4.5± 4.4
Human heart cells	0	0	55.7±10.1	18.1± 2.9	26.2± 7.2
	7.5	1.0	80.0± 6.6	12.8± 2.4	7.2± 4.2
Mouse kidney cells	0	0	25.3± 5.8	41.1± 8.1	33.6± 2.3
	0.5	1.0	97.3± 3.5	2.7± 3.5	0
Mouse macrophages	0	0	45.4± 9.7	34.0± 1.7	20.6± 8.0
	0.25	1.0	83.5± 3.2	14.8± 2.0	1.7± 1.2

*: Mean percentage was calculated from the results of five independent experiments.

†: Culture medium was Tc-199 containing 10% heat-inactivated calf serum.

Stewart, 1980). Neither inhibition of Tp multiplication in monocyte monolayers nor interferon activity in the assay was detected when normal beagle plasma was added.

HP-LKs were reconstituted with the medium + 40% CS to a concentration of 2, 1, 0.75, 0.5 or 0.25% and added to canine normal monocyte monolayers 1 hr after Tp inoculation to examine Tp multiplication. Cytotoxicity was observed when HP-LKs were added to a concentration of 2%, showing a noticeable destruction of monocyte

monolayers. When the concentration of HP-LKs was less than 1%, no cytotoxicity was noted, but Tp multiplication was inhibited strikingly. Only 0.9% of the cells contained 1~5 Tp, but no cells contained more than 6 Tp at all. The inhibitory effect of HP-LKs was reduced in parallel with decreases in the concentration of HP-LKs to 1, 0.75, 0.5 and 0.25% when Tp-containing cells were 0.9, 3.4, 7.6 and 11.2%, respectively. No interferon activity was detected in any of these concentrations of HP-LKs.

As shown in Table 3, the inhibitory effect of HP-LKs on Tp multiplication was investigated in heterologous cells. HP-LKs added to a concentration of 0.75% inhibited Tp multiplication in bovine monocytes and 17.6% of them contained Tp. No Tp multiplication was inhibited in HP-LKs-free cultures in which 70.3% of the cells contained Tp. Partial inhibitions of Tp multiplication were also found in human heart cell and mouse kidney cell monolayers. Cytotoxicity was rarely observed in homologous cells upon the addition of HP-LKs to a concentration of 1%. On the contrary, cytotoxicity was observable in heterologous cell cultures regardless of the concentration of HP-LKs added.

Discussion

From the results obtained, the existence of an LK-like substance in Tp-immune beagle plasma and the inhibiting activity of HP-LKs against Tp multiplication in vitro not only in homologous but also in heterologous cells were suggested confidently. This is probably because the polypeptides were cut to their low-molecular components by hydrolysis. When cultured in vitro with TLA, Con A or PHA, spleen cells from animals hyperimmune to Tp produce a supernatant termed LKs which contain MIF, MAF, interferon, Toxo-GIF and other similar factors (Freshman *et al.*, 1966; Sethi *et al.*, 1975; Shirahata *et al.*, 1975, 1977; Chinchilla and Frenkel, 1978; Igarashi *et al.*, 1979; Nagasawa *et al.*, 1980). Toxo-GIF or Toxo-GIF-like substance together with gamma interferon have been detected in the circulating blood of hyperimmune dogs after intravenous injection of TLA (Takei *et al.*, 1981).

The activity of LKs produced by Tp-immune spleen cells from one animal species is usually most effective against cells from the same species (Nagasawa *et al.*, 1980). This species specificity was also recognized in the plasma of a hyperimmune dog (Takei, unpublished data). This phenomenon of species specificity is quite unfavorable for the protection against infections since it indicates that only the homologous species preparation is expected to display a therapeutic value. But, HP-LKs activity was higher in homologous cells than in heterologous cells tested. In addition to anti-*Toxoplasma* activity, cytotoxicity was observed *in vitro* regarding both heterologous and homologous cells after HP-LKs were added to a high concentration.

The present preparation of HP-LKs contained 5 components of different molecular weight. The main component of the 5 had a calculated molecular weight of about 3,600. This variety in the components may have exerted various effects, including both anti-*Toxoplasma* and cytotoxic effects on the cells. This may be an alternative explanation for the close association between Toxo-GIF and cytotoxicity observed in the experiment. Therefore, if any component exerting cytotoxicity is clarified or removed, and HP-LKs are purified to a further extent by means of any method, such animal preparations as HP-LKs could be used for the promotion of microbicidal actions of concerning human cells. The effect of HP-LKs remains to be analyzed and confirmed. The authors are currently testing HP-LKs derived from other animal species for toxo-

plasmacidal effect on human heterologous cells.

Summary

This study deals mainly with some biologically active substances derived from hydrolyzed *Toxoplasma*-immune canine plasma (HP-LKs) and effects on the growth of the parasites not only in homologous but also in heterologous cells. The HP-LKs strongly inhibited *Toxoplasma* multiplication in homologous cells showing no cytotoxicity when administered at a concentration of 1% or less. The substance(s) also inhibited *Toxoplasma* multiplication in monolayers of heterologous mouse macrophages, mouse kidney cells, bovine monocytes and human heart cells. This ability of inhibiting *Toxoplasma* multiplication in homologous and heterologous cells was derived not from gamma type of interferon, but from Toxo-GIF or a Toxo-GIF-like substance in the plasma.

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加水分解トキソプラズマ免疫犬血漿の異種細胞内トキソプラズマ増殖抑制作用

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トキソプラズマ（Tp）免疫ビーグルに特異抗原を静脈注射し，24時間後に血液を採取し血漿を分離した．本血漿は Tp 抗体価 1：1,000 以上，および γ-インターフェロン活性 1：32 を示した．この血漿を酵素および酸・アルカリ加水分解，熱処理して上清を集め，Sephacryl S-200 および Toyo pearl HW-40 を用いてゲル濾過し，分子量 3,000～5,000 の画分を集めて，HP-LKs と名付けた．

Tp 接種1時間後の犬単球単層培養に，HP-LKs（0.25～2.0%）含有培養液を添加して48時間目の細胞内 Tp 増殖の有無を検討した．その結果，Tp 免疫血漿および 1% HP-LKs 添加群では，正常ビーグル血漿添加群に比較して，細胞内 Tp 増殖は顕著に抑制された．2% HP-LKs 添加群では，犬単球は障害された．

犬血漿由来 HP-LKs に対する異種細胞，ウシ単球，ヒト心筋細胞およびマウス腎細胞内 Tp 増殖に関しても，HP-LKs 添加によって細胞内 Tp 増殖は明らかに抑制された．この HP-LKs 中には γ-インターフェロン（免疫インターフェロン）活性は検出されなかった．