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Research Article

Pathological and Immunohistochemical Studies in Rabbits Experimentally Infected with *Toxoplasma gondii*

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Abstract: Experimental toxoplasmosis was induced in rabbits using different routes. A total of 42 animals were used; 36 of them were in the experimental groups and the other six comprised the control group. In addition, five guinea pigs were used for preparing hyperimmune sera against *Toxoplasma gondii*. The Ankara strain was inoculated intravenously (group I, n: 12), intraperitoneally (group II, n: 12) and intradermally (group III, n: 12) into the rabbits. Group IV animals were kept as controls (n: 6). Clinical and macroscopical findings varied according to the routes of inoculation and organs affected. Microscopically, multifocal necrosis with *T. gondii* tachyzoites was the main lesion in group I. In group II, necrotic lesions started at the serosal surfaces of the abdominal organs. In both groups, lesions were distributed in and invaded the deep parts of organs in accordance with post-inoculation days. In group III, lesions characterized by chronic inflammatory changes and tissue cysts were detected in the brain, liver and spleen in particular. Immunohistochemically, it was observed that positive reactions were not only observed in the tachyzoites and tissue cysts but also detected in antigenic clusters of destroyed organism on the serosal surfaces of the organs in the abdominal cavity.

Key Word: Experimental toxoplasmosis, immunohistochemistry, pathology, rabbit

Toxoplasma gondii ile Deneysel Olarak Enfekte Edilen Tavşanlarda Patolojik ve İmmunohistokimyasal Çalışmalar

Özet: Deneysel toksoplazmozis tavşanlarda farklı yollar kullanılarak oluşturuldu. Toplam 42 hayvanın kullanıldığı çalışmada 32 hayvan deney gruplarında arta kalan 6 hayvan da kontrol gruplarında idi. Ayrıca 5 kobaydan *Toxoplasma gondii*'ye karşı hiperimmun serum elde edildi. Ankara suşu tavşanlara intravenöz (grup I, n:12), intraperitoneal (grup II, n:12) ve intradermal (grup III, n:12) yollarla verildi. Grup IV'ü (n: 6) ise kontrol hayvanları oluşturdu. Klinik ve makroskobik bulgular inokulasyon yoluna ve etkilenen organlara göre farklılık gösterdi. Mikroskobik olarak, grup I'de *T. gondii*'nin takizot formlarının bulunduğu multifokal nekroz temel bulgu idi. Grup II'de nekrotik lezyonlar abdominal organların serozal yüzeyinden başladı. Lezyonlar her iki grupta da inokulasyon sonrası günlere bağlı olarak yayılım ve derinlik gösterdi. Grup III'de lezyonlar kronik yangısal değişikliklerle karakterizeydi ve doku kistleri özellikle beyin, karaciğer ve dalakta gözlendi. İmmunohistokimyasal olarak, pozitif reaksiyonlar sadece takizoitler ve doku kistlerine karşı değil aynı zamanda abdominal boşluktaki organların serozal yüzeylerinde yıkımlanmış organizmaların antijenik yapılarına karşı da dikkati çekti.

Anahtar Sözcükler: Deneysel toksoplazmozis, immunohistokimya, patoloji, tavşan

Introduction

Infection with *Toxoplasma gondii* is widely prevalent in many species of warm-blooded animals including humans (1). Epizootic toxoplasmosis has been reported in wild and domestic rabbits from many countries such as England (2), Argentina (3), Italy (4), Scandinavian countries (5,6) and the USA (7). However, experimental studies on *T. gondii* infections in rabbits are relatively

limited and the inoculation routes of the organism are mainly peroral (8,9) and subcutaneous (10,11). Furthermore, demonstration of *T. gondii* in rabbits' tissues except the liver (6) has not been reported using the immunoperoxidase technique.

The purposes of the present study were to observe the lesions occurring in experimental toxoplasmosis in rabbits using different routes (intravenous, intraperitoneal and intradermal) and to demonstrate different forms of organism in rabbit tissues.

Materials and Methods

Animals: New Zealand rabbits, 2-3 months old weighing 1.0-1.5 kg were bred in our animal unit. The sera of the rabbits were checked against toxoplasma antibody using the Sabin Feldman test (SF). Only those rabbits having no toxoplasma antibody were used in the experiment. A total of 42 rabbits were used; 36 of them were allocated to the experimental groups and the other six comprised the control group.

In the preparation of the primary antibody, guinea pigs (n: 5) obtained from Hıfzıssıhha Institute, Ankara, were utilized and they were free from toxoplasma antibody. Both groups of animals were fed *ad libitum* with commercial pellets (Ankara Feedstuff Industry, Turkey) lettuce, cabbage and carrot.

Toxoplasma: The Ankara strain (12) was used for infecting rabbits and for antibody production. It was maintained twice weekly by intraperitoneal passage in mice. This Ankara strain was diluted with 0.009% saline solution up to 40 tachyzoites per microscope field at a magnification of 40 and its concentration was 1 X 10^6 tachyzoites per ml. This solution was inoculated intravenously (0.5 ml into v. jugularis), intraperitoneally (1.0 ml) and intradermally (1.0 ml into regio fossa paralumbalis) into the rabbits.

Preparation of the primary serum: The Ankara strain was diluted with Freunds' complete adjuvant up to three tachyzoites per microscope field at a magnification of 40 (7.5 X 10^4 tachyzoites per ml) and inoculated subcutaneously as the first inoculation (1.0 ml) into the right hindlimb foot pad of the guinea pigs. The second inoculation was performed 4 d later. At this time, the inoculum included five tachyzoites per microscope field (1.25 X 10^5 tachyzoites per ml) at the same magnification, and 0.2 ml was inoculated into the animals, subcutaneously. Seven days later, the third inoculation was performed with 0.1 ml subcutaneously and 0.1 ml intramuscularly (m. gluteus). Seven tachyzoites (2 X 10^5 tachyzoites per ml) were present in this inoculum. The last inoculation was performed 7 d later with 0.1 ml subcutaneously and 0.1 ml intramuscularly. There were 10 tachyzoites (2.5 X 10^5 tachyzoites per ml) in this inoculum. Three days later, blood samples were taken from the animals, and sera were obtained and purified using sephadex column. The serum titer was measured using SF (1/128). These sera were kept at -20 ºC in a deep freeze.

Experimental design: The experiment involved four groups (Table 1), (group I, intravenous; group II, intraperitoneal; group III, intradermal; and group IV,

Table 1. Experimental design.

Post Inoculation Dates	Group I Number of animals Sacrificed	Group II Number of animals Sacrificed	Group III Number of animals Sacrificed	Group IV Number of animals Sacrificed
4th Day	3	2	-	-
5th Day	2	-	-	-
6th Day	2	3	-	-
7th Day	2	3	-	-
8th Day	3	2	-	2
10th Day	-	2	3	2
15th Day	-	-	3	-
30th Day	-	-	3	-
40th Day	-	-	3	2
Total	12	12	12	6

control). For each group 12 animals were used except for group IV. In group IV six animals were used as controls. Animals that died spontaneously were excluded from the experiment. Therefore, there were differences in the number of animals in each group. After the inoculation, in group I on the 4th and 8th days three animals and on the 5th, 6th and 7th days two animals were sacrificed; in group II on the 4th, 8th and 10th days two animals, and on the 6th and 7th days three animals were sacrificed; and in group III on the 10th, 15th, 30th and 40th days three animals were sacrificed. As presented in Table 1, in group IV on the 8th, 10th and 40th days, which match the end of each experiment group, two animals were sacrificed. Throughout the experiments the ethical rules of the "Animal Experimentation of University of Ankara, Faculty of Veterinary Medicine" were followed.

Pathological examination: Sacrificed animals were necropsied and macroscopical findings were recorded. Then tissue samples were fixed in 10% neutral buffered formalin, after routine procedures the samples were embedded in paraffin wax, sectioned at 5-6 µm and stained with hematoxylin and eosin (H-E). Some of the selected sections were also stained with periodic acid Schiff (PAS). The sections were examined under a light microscope and the lesions were scored according to the spread and severity of the necrotic lesions.

Immunohistochemical examination: The avidin-biotin-peroxidase complex (ABPC) test was performed according to the manufacturer's instructions (Shandon Inc., Cadenza tag, Pittsburg PA). After deparaffinization, the endogenous peroxidase activity of each tissue was blocked with 0.3% H_2O_2. Then sections were incubated sequentially with normal goat serum, primary serum, biotinylated anti-guinea pig IgG goat serum and ABC reagent. The peroxide was located with 0.050 ml acetate buffer, 0.025 ml 3-amino-9 ethylcarbazole (AEC) chromogen and 0.025 ml 3% H_2O_2 in 4 ml deionised water. The sections were counterstained with Meyer's hematoxylin. The reagents for the ABPC test (except primary serum) were purchased commercially. Guinea pig immune serum against *T. gondii* was used as a primary serum at a dilution of 1/64. As a control, normal guinea pig serum and phosphate buffered solution (PBS) were used for the first antiserum.

Results

Clinical Findings

These findings differed depending on the routes of inoculation and affected organs. However, affected animals lost their appetite and deteriorated showing fever, anorexia, lethargia, nasolacrimal discharges and respiratoric disorders. Other clinical findings included tremor, uncoordinated gait, voluminous hair coat and diarrhea. The illness duration was 8 d in group I and group II. In group III, animals did not exhibit any clinical findings except for the wound at the inoculation site. No clinical findings were observed in group IV.

Pathological Findings

Macroscopical: There was a correlation between post-inoculation days and lesions. In group I, the most characteristic lesion was pale yellow necrotic foci 1-2 mm in diameter. These foci were distributed diffusely all over the liver and spleen. The same lesions were also observed in one heart (case no. 6). Moreover, focal pneumonic areas at the cranial lobes of the lungs, subcapsular hemorrhages and pale areas in the kidney were other lesions. In group II, cloudy yellowish fibrinous exudates in various amounts (10-100 ml) were detected in the abdominal cavity. These exudates covered all of the abdominal organs and serosal surfaces. For this reason, all abdominal organs were cloudy in appearance, and also subcapsular located necrotic foci were found in these organs. In addition, subcapsular petechial hemorrhages and areas pale in color were observed in the kidney. In some cases (case nos. 14, 16, 24), small pneumonic areas were found in the cranial lobes of lungs. In group III, necrotic lesions 1.5-3.0 cm in diamater were seen at the inoculation site, and enlargement was observed, especially in inguinofemoral lymph nodes draining the inoculation sites, and most of them were necrotic in appearance at the cut surfaces. Clear fluids in 5-10 ml in the abdominal cavity were also found in some cases (case nos. 25, 31).

Microscopical: All findings are shown in Tables 2-5. In group I, lesions were mainly observed in the liver and spleen, and they were focally distributed in the organs. *Toxoplasma gondii* tachyzoites were not seen until the 4th day after inoculation in the severe focal necrotic areas. Lesions increased gradually 5, 6, 7 and 8 d after inoculation and inflammatory cell types changed into mononuclear series cells. Lesions were not detected in the

Table 2. Localization of the histopathologic findings.

Case No	Group	Post-Inoculation Days	Liver	Spleen	Lungs	Lymph Nodes	Brain	Heart	Intestines	Stomach	Pancreas	Kidney	Urinary Bladder	Skin
1	IV	4	++	++	+	+++	-	-	++	-	-	+	-	-
2	IV	4	+++	+	++	+	++	-	-	-	-	+	-	-
3	IV	4	++	+	+	-	+++	-	-	-	-	+++	-	-
4	IV	5	++	-	+	++	-	-	-	-	-	-	-	-
5	IV	5	+++	++	-	-	+++	-	-	-	-	+++	-	-
6	IV	6	+++	++	+++	++	++	++	+++	-	-	-	-	-
7	IV	6	+++	++	+++	++	+	-	+	-	-	+	-	-
8	IV	7	+++	-	++	+++	++	-	+	-	-	-	-	-
9	IV	7	+++	+++	-	-	+	-	-	-	-	+	-	-
10	IV	8	+++	+++	-	+	-	-	-	-	-	+++	-	-
11	IV	8	+++	++	++	++	-	-	++	-	-	-	-	-
12	IV	8	+	-	+	+	+	-	-	-	-	-	-	-
13	IP	4	++	+++	-	+++	+	-	+++	-	++	-	-	-
14	IP	4	++	+	++	++	-	-	+	-	-	-	-	-
15	IP	6	+++	+++	-	+++	+	-	+++	+++	++	++	-	-
16	IP	6	++	+++	++	+++	-	-	+++	+++	+	+	+++	-
17	IP	6	+++	+++	+	++	+	-	+++	-	++	-	-	-
18	IP	7	+++	+++	-	+++	-	+	+++	-	-	-	-	-
19	IP	7	++	+++	-	++	+	-	++	+	++	-	-	-
20	IP	7	+++	+++	+	++	-	++	+++	-	-	-	-	-
21	IP	8	+++	-	-	+++	-	-	-	-	-	-	-	-
22	IP	8	++	++	+	++	++	-	+	-	+	+	+++	-
23	IP	10	+++	++	-	-	-	-	+++	-	+++	-	-	-
24	IP	10	++	+++	++	++	++	-	-	-	-	-	-	-
25	ID	10	++	++	+++	++	-	-	++	-	-	-	-	+++
26	ID	10	+	-	-	+++	+	-	+	-	-	+	-	+++
27	ID	10	++	-	-	+++	+	-	-	-	-	-	-	+++
28	ID	15	+	++	+	++	+	+	++	-	-	+	-	+++
29	ID	15	+	+	+	+	+	-	-	-	-	+	-	+++
30	ID	15	+	-	-	+++	+	+	-	-	+	-	-	+++
31	ID	30	-	+	-	+	-	-	-	-	-	+	-	+
32	ID	30	-	+	-	+	++	-	+	-	-	-	-	+
33	ID	30	+	-	-	+	+++	-	+++	-	-	-	-	+++
34	ID	40	+	-	+	+	+++	-	+++	-	-	+	-	+++
35	ID	40	+	+	-	-	++	+	-	-	-	-	-	+++
36	ID	40	+	-	+	++	++	-	-	-	-	-	-	++

Table 3. Severity of the histopathologic lesions in group I.

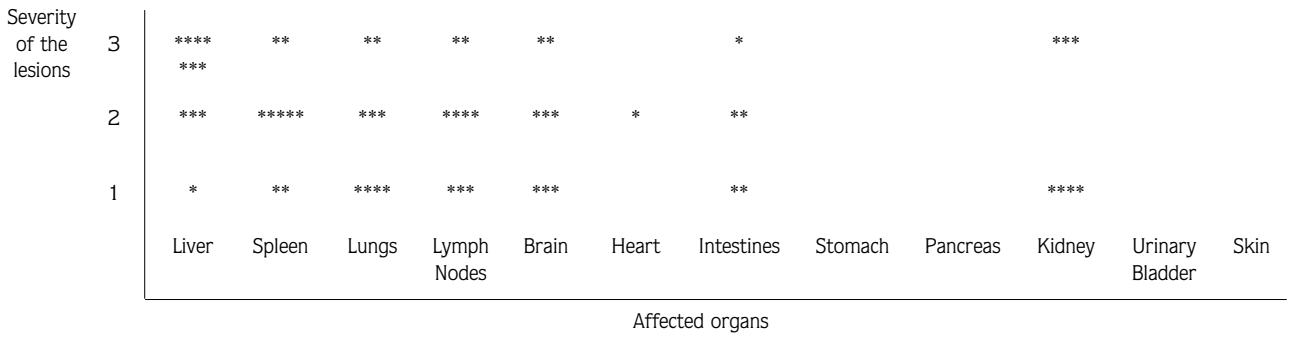

Table 4. Severity of the histopathologic lesions in group II.

Severity of the lesions	Liver	Spleen	Lungs	Lymph Nodes	Brain	Heart	Intestines	Stomach	Pancreas	Kidney	Urinary Bladder	Skin
3	**** **	***** ***		*****			*******	**	*		**	
2	*** ***	**	***	**** **	**	*	*		***	*		
1		*	***		*	*	**	*	**	**		

Affected organs

Table 5. Severity of the histopathologic lesions in group III.

Severity of the lesions	Liver	Spleen	Lungs	Lymph Nodes	Brain	Heart	Intestines	Stomach	Pancreas	Kidney	Urinary Bladder	Skin
3			*	***	**		**					**** **** *
2	**	**		***	***		**					*
1	*** ** ***	****	****	*****	**** *	***	**		*	**** *		*

Affected organs

kidney (case nos. 4, 6, 8, 11, 12), brain (case nos. 1, 4, 10, 11), spleen (case nos. 4, 8, 12), lungs (case nos. 5, 9, 10) or lymph nodes (case nos. 3, 5, 9), but focal lesions in the heart (case no; 6) and intestines (case nos. 1, 6-8, 11) were seen. In the lungs, lesions were located around the vessels, and were characterized by focal interstitial pneumonia (Figure 1-b).

In group II, the necrotic lesions started on the serosal surfaces of the abdominal cavity organs (Figures 1-a,c), and according to the duration of the experiment by means of PIDs, they invaded the deep parts of these organs. Again inflammatory cells changed to mononuclear cells 7 d post-inoculation. Lesions in this group were mainly located in the liver, spleen and serosal surfaces of the abdominal cavity (intestines, stomach, pancreas, urogenital system) and were occasionally seen in the interstitial tissues of the pancreas. Lung lesions were observed in three cases (case nos. 16, 17, 22), and very slight lesions were found in the brain (Figure 1-d) (case nos. 13, 15, 17, 19, 22) and kidney (case nos. 15, 16, 22). In group III, large necrotic areas were found, especially in the lymph nodes draining inoculation sites, and hyperplastic changes were also observed. Lesions were not observed in the spleen (case nos. 26, 27, 30, 33, 34), liver (case nos. 31, 32), brain (case nos. 25, 31) or lymph node (case no. 35). Granulomatous foci including mainly mononuclear cells were seen in the liver and spleen. Lesions characterized by chronic inflammatory changes and tissue cysts (bradyzoites) were detected in the intestine (case nos. 25, 26, 28, 31, 33, 34), kidney (case nos. 26, 28, 29, 31, 34), lung (case nos. 25, 28, 29, 34), heart (case nos. 28, 30, 35) and pancreas (case no. 30). Lesions in the lung were characterized by focal interstitial pneumonia; however, in the heart *T. gondii* tissue cysts were observed without inflammatory changes. In the intestine necrotic foci were found in the submucosa, in the pancreas and kidney focal neutrophilic and necrotic lesions were found on

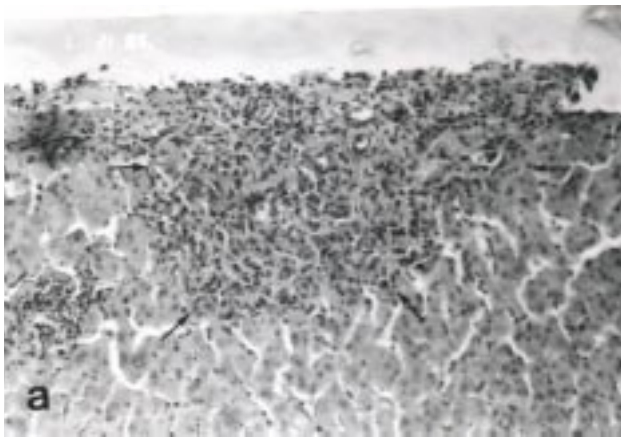

Figure 1 a) Large necrotic area starting from the serosal surface of the liver invaded the parenchyma (arrows), HE x 160.

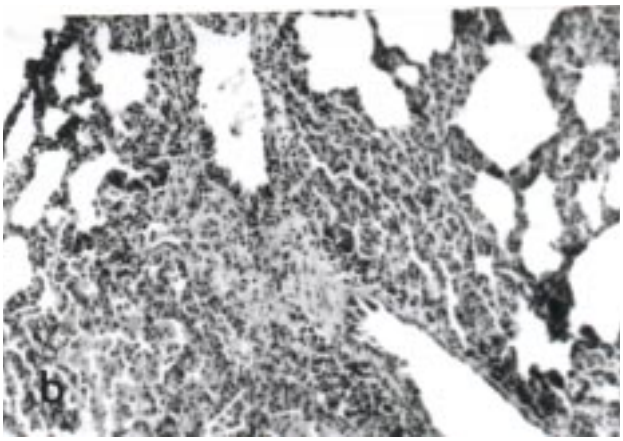

Figure 1. b) Focal interstitial pneumonia, HE x 160.

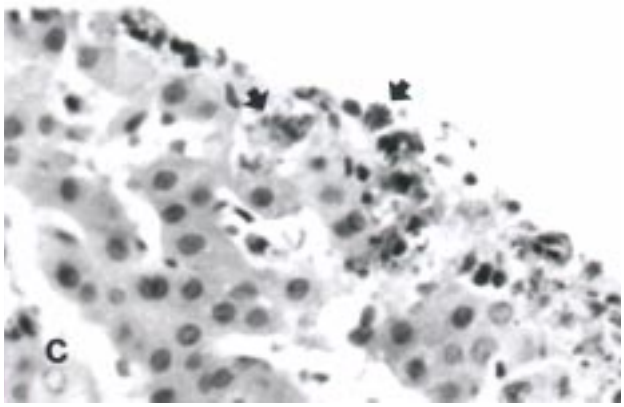

Figure 1. c) Necrosis on the serosal surface of the liver. Note the free tachyzoite groups (arrows), HE x 640.

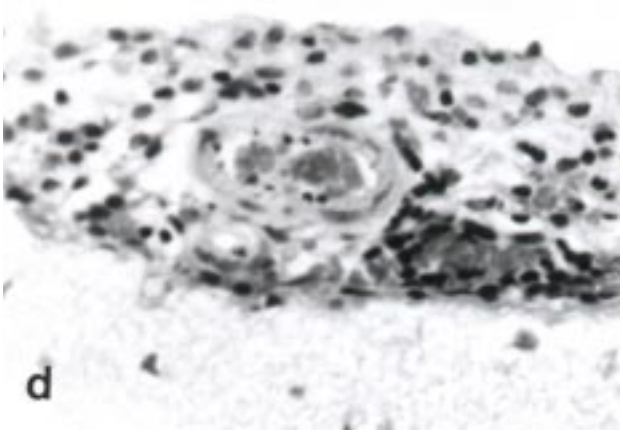

Figure 1 d) Meninges have thickened with mononuclear cells, HE x 320.

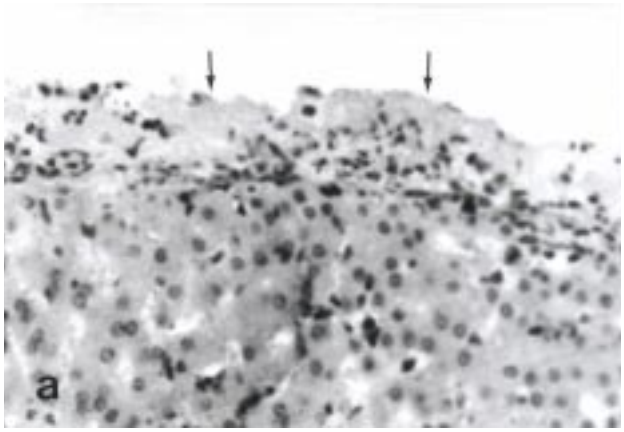

Figure 2. a) Slight immunoreactive materials (arrows) on the serosal surface of the liver. Immunoperoxidase - Meyer's hematoxylin x 320.

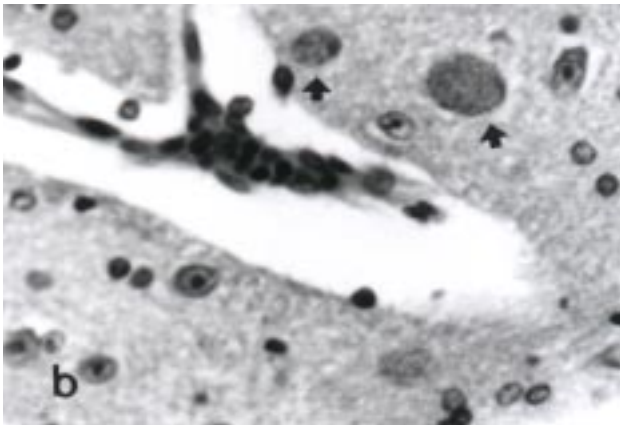

Figure 2. b) *Toxoplasma gondii* tissue cysts (arrows) without an inflammatory reaction in the brain. Immunoperoxidase - Meyer's hematoxylin x 640.

interstitial tissues, and in the brain granulomatous inflammatory foci including mainly mononuclear cells located at the telencephalon and mesencephalon were found. *T. gondii* tissue cysts were seen near these lesions. There were no macroscopic or microscopic findings in the control animals.

Immunoperoxidase Findings

The H-E stained tissue sections containing *T. gondii*, which were examined in the ABPC test, reacted positively with *T. gondii* antiserum but not with PBS or the normal guinea pig serum examined. Positive reactions as shown by a deep brown color were observed in tissue sections prepared from organs. The results were negative when normal guinea pig serum and PBS were used as a primary serum. It was noted that positive reactions were not only observed in the tachyzoites and bradyzoites but also in antigenic clusters of the organism destroyed on the serosal surfaces of the organs such as the intestine, stomach and pancreas in group II. Bradyzoites were usually stained less strongly than tachyzoites. There was also some slight staining of the cyst wall. In addition, necrotic material and structures in the cytoplasm of macrophages were stained occasionally. Despite the blocking step with normal goat serum, some of nonspecific staining of connective tissue persisted. However, this staining could be differentiated from specific staining and did not confuse interpretation. Nonspecific staining was reduced when the washing period was extended at the last step of the staining procedure. In group I, antigenic positive reactions were obvious near the necrotic lesions. On the other hand, these positive reactions were seen in the visceral layer of the peritoneum that covered the abdominal cavity's organs in group II (Figure 2-a). In group III, positive staining was found, especially in the tissue cysts near the granulomatous lesions on the brain (Figure 2-b), liver, spleen, lungs and heart.

Discussion

There are several reports related to toxoplasma outbreaks in wild and domestic rabbits (2,3,7,13,14). Severe acute toxoplasmosis was observed in these outbreaks, and many animals died. In addition, it was reported that most outbreaks occurred in winter months (January and February) and few in summer months (June and July). On the other hand, acute systemic toxoplasmosis is seen especially in young and immunsuppressed animals (1,15). In the present study, we also used young animals and acute systemic toxoplasmosis occurred in groups I and II. However, in group III, because of the intradermal inoculation, we achieved successful chronic or inactive toxoplasmosis characterized by tissue cysts of the organism.

The multifocal necrosis in acute toxoplasmosis, especially in the lymph nodes, spleen and liver, is an obvious finding in rabbits naturally or experimentally infected with *T. gondii* (2,3,7,11,13,14). Besides these organs, lesions have been observed in the lung (2,4,13,16) and heart (10,17,18). In our study, lesions were detected in similar locations, but the inoculation route caused differences in the occurring lesions.

Lesions in the visceral organs of rabbits have been seen 1-2 weeks after organisms were given perorally (1,15). In the present study, the period when the organisms were given intravenously, intraperitoneally and intradermally was 4, 4 and 10 d, respectively. In groups I and II, lesions were multifocal necrosis and *T. gondii* tachyzoites near these areas, whereas they were mainly located in the liver, brain and lungs as a granulomatous characteristic with *T. gondii* tissue cysts in group III. It supposed that this situation was directly related to the inoculation routes. In the experimental infections, the virulence of the agent was also important (10,11), but this subject was not examined.

In the diagnosis of toxoplasmosis, there are several tecniques including serology (SF, HI) and pathology (histopathology, ultrastructure, immunohistochemistry). *Toxoplasma gondii* tissue cysts are easily visualized by a variety of routine histological staining methods; however; tachyzoites are difficult to recognize in histologic preparations of materials when using conventional techniques. On the other hand, the microscopic appearance of several protozoans (sarcocystis, *Neospora caninum*, leishmanial forms of *Trypanasoma cruzi*, *Besnoitia besnoitii, Hammondia hammondii*) are very similar to toxoplasma, and they must be differentiated from toxoplasma, even though there are some morphologic differences (1,5,15,19).

Toxoplasma gondii has been detected using the immunoperoxidase technique in tissue sections of humans (20), pigs (21), sheep (22), cats (5,23), dogs, blue foxes and rabbits (5,6). In rabbits, *T. gondii* has been

demonstrated only in the liver using the peroxidase-antiperoxidase (PAP) method, and also this method has been applied retrospectively on the storage materials confirming the routine histopathologic diagnosis of toxoplasmosis (6). In the present study, we achieved experimental toxoplasmosis using different routes and demonstrated different forms of organisms and antigenic clusters in rabbit tissues using the ABPC test.

The less intense staining of the bradyzoites compared with the tachyzoites of *T. gondii* may be due to antigenic differences. Lunde and Jacobs (24) reported that antisera against tachyzoites of *T. gondii* reacted only partially with bradyzoites, while antisera against bradyzoites did not crossreact at all with tachyzoites. Hyperimmune sera made by the inoculation of tachyzoites alone as in the present study may contain antibodies almost exclusively against tachyzoites, since the antigens of later developing bradyzoites may not be accessible to the immune system because of sequestration inside the cyst wall. Another possibility for these different reactions may be that because bradyzoites are tighly packed and surrounded by a dense material, the antigens are rendered less available for antibodies in immunological tests.

In group II, the positive staining along the serosal surfaces of the abdominal cavity's organs could suggest the presence of antigenic deposits derived from the organism. The cyst wall of *T. gondii* is thought to be formed by deposition of material from the organism on the membrane of a parasitophorous vacuole (1), and might therefore contain parasitic antigens.

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