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Response of White Mice to Inoculation of Irradiated Organisms of the *Toxoplasma* Strain RH

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Summary. Chemotherapeutic agents available for use against toxoplasmosis are usually not suitable for prophylactic purposes because of their toxicity. The observed increasing number of activated latent infections with *Toxoplasma*, especially in immune suppressed patients, requires that safe techniques are available for use during the patients' regression period.

Pretreatment of mice with *Toxoplasma* killed by irradiation appeared to induce resistance to challenge with virulent organisms. Survival times of six months have been observed to date. Increasing effectiveness was seen after more than one administration.

Further investigation into the duration of effective resistance is needed; the question of at which intervals subsequent inoculations should be performed in order to acquire a booster effect, if any, has still to be solved before application to man can be recommended.

Introduction

People with a deficient immune response, whether due to an underlying illness or immune suppressive treatment, are much more susceptible to infectious agents than are those people with a normal immune mechanism. The risks are twofold: reactivation of a latent infection as well as illness caused by an agent from the environment. This knowledge was the stimulus for the search for preventive measures against such risks.

Toxoplasma, an ubiquitous protozoan, has increasingly become known as one of the manifold organisms which may threaten the compromised host. This observation was emphasized by Ruskin and Remington (1976) when listing 83 cases of toxoplasmosis along with the underlying illness from 1950 until May 1975.

If toxoplasmosis is definitely established in the course of an immune suppressive treatment, pyrimethamin combined with sulfapyrimidines are the chemotherapeutic agents of choice. Unfortunately, their toxicity may do still more

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harm to the patient who is already in a hazardous position. For the same reason, attempts to prevent toxoplasmosis by prophylactic treatment with these drugs must not be done. The need for a nontoxic treatment is evident.

In a latent phase of a *Toxoplasma* infection, the organism is present in the cystic form, causing no harm to the host; importantly, however, it stimulates a specific resistance in the host which is called premunition. When the immune response is suppressed by any means the balance is shifted to the host's disadvantage. Activating the immune response in a patient during the regression period would, in all probability, protect him. Furthermore, the absence of immunity to *Toxoplasma* in the child-bearing age may, in certain cases, make preventive measures desirable. Avoidance of consuming insufficiently cooked meat and the handling of cat droppings are matters of routine. The administration of a vaccine which does no harm to the fetus would then be appropiate.

In experimental toxoplasmosis, prophylactic treatment with organisms killed by heating or freezing and with specific antibody containing blood serum prolonged the survival time but did not prevent death after inoculation with toxoplasmas of a virulent strain (unpublished observations). The effect of irradiated toxoplasmas for prophylactic purposes seemed worthwhile to investigate.

Lund et al. (1961), among others, studied the influence of irradiation on the propagation of *Toxoplasma* of the RH strain in cell cultures. Doses of 600 to 3000 rad had absolutely no effect on the penetration of cells and subsequent multiplication. When a maximum of 30,000 rad was used, the irradiated organisms appeared longer to no multiply, although abortive multiplications, never more than four parasites per clone, were observed in a few cases. Kobayashi and Jacobs (1964) demonstrated loss of infectivity of RH strain organisms when exposed to 15,400 rad. Seah and Hucal's actual aim (1974, 1975) was to prepare a vaccine from irradiated organisms of the RH strain. In the discussion, they express 'a suspicion that some irradiated parasites may continue to multiply in the vaccinated mice.' They did not mention the reason why animal passages were not done to verify their suspicion nor why serologic examination of blood from subinoculated mice was not performed.

In our opinion, it is of paramount importance to make absolutely certain whether the inoculum consisting of irradiated *Toxoplasma* still contains live organisms or not, whether with lowered or no virulence at all. For, any remaining live *Toxoplasma*, though damaged by irradiation, may not have completely lost its capacity for multiplication. Such an organism is a great risk to the host because a possible change in virulence by whatever cause has to be taken into account. Our efforts were fundamentally focused on preparing a vaccine without that risk.

Materials and Methods

SPF Swiss random bred female mice were used throughout the experiments. They were seven weeks of age when the experiments were begun, weighed about 18 g, and were obtained from the Central Institute for The Breeding of Laboratory Animals, Zeist, The Netherlands.

For irradiation, a Philips Co-60 XK-5100 was available[1]; it was equipped with a cylindrical X-ray source of 2 cm in diameter, delivering 258 rad per minute with a distance of 40 cm to

[1] By courtesy of Dr. J.H. Mellink, Faculty of Medicine, State University Leyden, The Netherlands

the object to be exposed. An aluminium plate on the covered plastic Petri dish ensured an equal distribution of the X-rays among the toxoplasmas within. A height of one cm of the column of the suspension was not exceeded (at 3 cm below the surface, absorption was about 90%).

The *Toxoplasma* strain RH was used in batches recovered from the peritoneal cavity of mice inoculated two days previously. To a mixture of equal volumes of *Toxoplasma* containing peritoneal exudate and a solution of physiologic saline heparin was added (10 iu per ml). Afterwards, physiologic saline alone served as the diluent. The parasites were counted in a Bürker counting chamber before and after X-ray treatment.

When no *Toxoplasma* could be demonstrated in the peritoneal exudate or in the brain material of a mouse, material from brain, heart, liver, spleen, and saline suspensions of contents of the peritoneal cavity were subinoculated when irradiated *Toxoplasma* organisms had been administered; otherwise, only brains were examined.

All inoculations into mice were done intraperitoneally. For demonstration of *Toxoplasma*, fresh and/or Giemsa stained slides were examined. The Sabin-Feldman-dye-test (DT) was used for detecting antibodies to *Toxoplasma*. A titer of $<{}^1/_4$ (final dilution) was considered negative.

The response of mice following inoculation of irradiated toxoplasmas was compared with that of those inoculated with nonirradiated organisms from the same batch and otherwise manipulated in the same way. A second and third inoculation with irradiated toxoplasmas, meant boosters, were checked in the same manner. When pretreated mice were challenged, the inoculum contained 10^4 *Toxoplasma* organisms and was checked by inoculation into non-pretreated animals.

The number of control mice was either the same or, when smaller, never less than half of the test animals.

Spontaneous infection with *Toxoplasma* was checked for in every group of mice to be used in the respective experiments by examining random samples. If the dye test titer was $\geqq{}^1/_4$, brains were inoculated into a new mouse.

Initial Experiment

This was an investigation into the suitable number of toxoplasmas to be exposed to such a dose of X-rays that the loss of multiplication capacity could be established when inoculated into mice.

Equal portions of suspensions of a known number of toxoplasmas per ml were exposed to different doses of X-rays. Groups of mice were inoculated with 10^4 and 10^6 organisms, respectively. Survivors among the mice were killed three weeks to nine months after inoculation for examination for the persistence of live *Toxoplasma* and DT antibodies.

Results

Inocula of both 10^4 and 10^6 toxoplasmas from portions exposed to either 5 or 10 Krad killed each of the groups of ten mice within seven to ten days. The control mice died five and seven days after inoculation with 10^6 and 10^4 nonirradiated toxoplasmas, respectively.

When doses of 15 and 20 Krad were used, survival of over three weeks (first examination) and up to nine months (last examination) was established. The number of mice involved is shown in Table 1, along with the results of the attempted isolations from several organs and those of the DT.

Conclusions

Doses of 5 and 10 Krad appeared to be insufficient with respect to the killing effect on *Toxoplasma* organisms, while 15 and 20 Krad were found to be reliable.

Table 1. Number of mice examined three weeks to nine months after inoculation of irradiated toxoplasms

Irradiation in Krad	15	20	15	20	20
Number of toxoplasms per inoculum	10^4		10^6		2×10^6
Number of inoculations					
1	11	11	13	6	8
2				6	6
3				4	4
	11	11	13	16	18 (total 69)

Notes: 69 unsuccessful attempts to isolate Toxoplasma. 18 mice negative in the DT: $18 \times$ inoculum of 10^4 toxoplasms. 51 mice positive in the DT: titres ranging from $1/4 < T < 1/16384$. $47 \times$ inoculum of 10^6 or 2×10^6 toxoplasms. $4 \times$ inoculum of 10^4 toxoplasms

Fifty-one positive DT's out of 69 cases may be considered as proof of successful administration of antibody inducing *Toxoplasma* antigens when irradiated organisms were inoculated (see notes Table 1).

In attempts to isolate *Toxoplasma* from mice treated with irradiated organisms, subinoculation of brain material only is justified, in view of the simultaneous negative results obtained with several other organs, including contents of the peritoneal cavity.

Main Experiment

This was an investigation into the survival of mice after challenge with virulent toxoplasmas when pretreated once, twice or three times with irradiated organisms.

Groups of mice were challenged with 10^4 virulent toxoplasmas at intervals of one week or longer after the last inoculation with organisms from batches exposed to 15 and 20 Krad of X-rays, according to the following working scheme shown in Table 2.

Table 2. Working scheme

Dose of X-rays in Krad	15	20	15	20	20
Number of toxoplasmas per inoculum	10^4		10^6		2×10^6
Number of treatments before challenge	1	1	1	1,2,3	1,2,3

Groups of non-pretreated mice inoculated with 10^4 virulent organisms served as controls.

Results

The results of the main experiment are shown in Table 3

Table 3. Survivors among mice when challenged with 10^4 virulent toxoplasmas after treatment with irradiated organisms (number examined in brackets)

Interval between treatment and challenge in weeks	Doses in Krad								
	15	20	15			20			
	Number of toxoplasmas per inoculum								
	10^4		10^6		10^6			2×10^6	
	Number of treatments								
	1	1	1	1	2	3	1	2	3
1	–	–	–	–	2(2)	2(2)	–	1(2)	2(2)
2	0(2)	0(2)	0(2)	3(4)	2(2)	2(2)	1(2)	2(2)	2(2)
3	0(3)	0(3)	2(3)	2(5)	1(2)	2(2)	1(2)	1(2)	2(2)
4	0(2)	0(2)	0(2)	2(4)	1(2)	–	1(2)	1(2)	–
5	0(3)	0(3)	0(3)	1(5)	–	–	0(2)	–	–
6	0(3)	0(3)	0(3)	0(5)	0(2)	–	1(2)	1(2)	–
7	0(2)	0(2)	0(2)	0(2)	–	–	–	–	–
8	–	–	–	–	–	–	1(2)	–	–
10	–	–	–	–	–	2(2)	–	–	2(2)
12	(3)	0(3)	0(2)	0(2)	0(2)	1(2)	–	0(2)	–
14	–	–	–	–	1(2)	–	0(2)	1(2)	–
	0(18)	0(18)	2(17)	8(27)	7(14)	9(10)	5(14)	7(14)	8(8)

At the time of the preparation of this paper, the survival time of mice under observation was six months after challenge, when they were killed for examination. These were mice challenged two, three and four weeks, respectively, after one pretreatment with 10^6 irradiated toxoplasmas exposed to 20 Krad (see Table 3).

Discussion

Intracellularly situated *Toxoplasma* organisms were not taken into account when irradiation was intended, as they would not be less exposed to X-rays (Mellink). By the time of the final count immediately before inoculation, the organisms could be supposed to be situated extracellularly, since about four hours had passed after collection of the toxoplasmas from mice; for this reason, the count was considered to be reliable.

Lack of knowledge as to what extent the biologic properties of *Toxoplasma* would be affected by irradiation under our conditions let to the search for live *Toxoplasma* in several organs of the mice after inoculation with X-ray treated organisms. This was done by subinoculation of organs, irrespective

of the DT results; otherwise killed *Toxoplasma* might also induce antibody formation, thus complicating the DT results. A negative DT might be obtained because of the possibly delayed antibody formation. Organisms might remain for a long time in the peritoneal cavity of the mouse because of possibly reduced motility; this is a reason to subinoculate peritoneal contents to trace them. For the same reason, not only brain but other organ material was subinoculated.

In two cases of random tests for spontaneous infection with *Toxoplasma* in mice, we found titers of $>{}^1/_4$, although $<{}^1/_{64}$ along with unsuccessful attempts to isolate the organism. These titer levels were therefore considered to be nonspecific. For mice subinoculated with organs from animals which had been treated with irradiated *Toxoplasma* organisms, positive DT's combined with unsuccessful isolation could be explained by antibodies transmitted together with traces of blood and/or induced by antigens from irradiated toxoplasmas.

Though examined at comparable times, inoculation of 10^4 irradiated toxoplasmas resulted in 18 negative DT's in mice (see Table 1, notes), while, when 10^6 organisms were inoculated, positive DT's were established in all mice. In all probability, the negative results are due to the fact that antibodies to *Toxoplasma* had already disappeared from the blood by the time of examination and are not due to unsuccessful inoculation of antibody inducing *Toxoplasma* antigens.

When four apparently healthy mice which had been pretreated with irradiated organisms were killed for examination six months after challenge, *Toxoplasma* which caused fatal infections when subinoculated into nonpretreated mice were isolated from two of them. An equilibrium between specific resistance of the host and virulence of the *Toxoplasma* strain is supposed to be brought about in the pretreated mice when challenged. Because of this, we do not consider the possibility of having isolated progeny of *Toxoplasma* from X-ray treated batches. This is supported by 51 cases with positive DT's along with unsuccessful isolation (see Table 1, notes). Besides, the positive results are proof of having actually inoculated *Toxoplasma* antibody inducing antigens.

Toxoplasma was not isolated from the remaining two mice. It is supposed that pretreatment induced specific resistance capable of even destroying virulent *Toxoplasma*. A possible failure in challenging is not likely, since all of the relevant control mice died of acute toxoplasmosis.

Since the duration of a supposedly effective specific resistance was unknown, the time of challenge after pretreatment was arbitrarily chosen, so were the intervals for the intended booster administrations.

Final Conclusions and Questions Still to be Answered

The response of mice to inoculation of irradiation-killed toxoplasmas of the virulent RH strain was manifested by survival when challenged with organisms of the same strain and by apparently complete recovery after passing through a critical period.

The effect was increased when more than one pretreatment was given. Under certain conditions, the supposedly induced resistance destroyed the *Toxoplasma*

organisms with which the mice were challenged; if they were not destroyed, the resistance appeared to be in balance with the virulence of the strain.

Undoubtedly, the experiments need extension in order to verify the results and findings up to now.

The duration of effectiveness of the induced resistance needs further investigation. This is attended by the question of at what intervals should subsequent treatment be given in order to achieve optimum specific resistance and whether in so doing a booster effect would be acquired. These questions obviously indicate that this method of vaccination in human medical practice would be premature at this time.

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