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EFFECT OF NEEM AND MANNOPROTEINS OF *CANDIDA ALBICANS* ON SOME IMMUNOLOGIC PARAMETERS ON MICE VACCINATED WITH BR. REV-1 VACCINE

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ABSTRACT
The study was conducted to evaluate the immunomodulator potentials of neem seed extracts (aqueous and ethanolic) and *C. albicans* cell wall mannoproteins in mice vaccinated with *Brucella* Rev-1 vaccine in relation to Prednisolone treatment. The study was included two main groups (160 mice for each), each group was divided into eight subgroups 20 mice each, subgroups treated as fallowing: I: treated with distilled water only, II: *Brucella* Rev-1 vaccine only,: III treated with mannoproteins only, IV: treated with aqueous extract only V: treated with ethanolic extract only respectively, last three groups VI, VII and VIII: treated with mannoproteins, aqueous and ethanolic extract, respectively, then they were vaccinated with *Brucella* Rev-1. The second eight sub groups were treated as an experiment number one and injected subcutaneously (0.2 ml) with the immunosuppressed drug prednisolone (5 mg/kg) 5 days prior to the treatment regimens to be effective in mediating immune suppression in mice. All these treatments were carried out on day 1 and then vaccinated with Brucella Rev-1 vaccine on day 4 in all mice. The mice were scarified and tested as follows, on day 10 after vaccination for nitro blue tetrazolium index (NTB), on day 14 for (delayed-type hypersensitivity reaction (DTH) and lymphocyte transformation test (LTT). The results demonstrated clear immunomodulator effects, the NBT index was significantly increased in immunomodulator-treated and vaccinated mice in comparison with control negative (I) and (II, III, IV and V) groups at the level (P≤ P0.05 and P≤ 0.01). The mitotic index of lymphocytes cells showed significant increase (P≤ 0.05 and P 0.01) percentage in animmunomodulator-treated and -vaccinated mice in comparison with negative and positive control groups. In DTH, index was significantly increased in immunomodulator-treated and -vaccinated mice in comparison with negative and positive groups, and a best result was observed in group IV, 24 hours post-brucelline injection, and in general the response after 24 hours was better than 48 hours. In this study immunomodulation could be concluded that neem seed extracts (aqueous and ethanol) and mannoproteins isolated from *Candida albicans* cell wall are an important immunomodulators in the development of immune response against *Brucellaabortus*, especially if they are employed in the vaccine strategy, against such important pathogen. From the present results, it is possible to conclude that the neem seed extracts (aqueous and ethanolic) and the cell wall mannoproteins of *C. albicans* might be a potential immune adjuvant for inducing active immunity against *brucella*, and may act as Immunopotentiators through increasing microsomal proteins. These proteins have a binding activity to antigens, and such binding helps in extending the half-life of the antigen by a gradual release over a long period.

KEY WORDS: Brucella vaccine, Neem, *Candida albicans*, Mice, *etc*.

INTRODUCTION

Materials of fungal and/or plant origins have been the interest of different investigators around the globe with their aims to establish the immunomodulator potentials of these materials. Some risks associated with attenuated or killed whole-organism vaccines can be avoided with vaccines that consist of specific purified macromolecules derived from Pathogens or in combination with plant materials (1and 2).*Candida albicans* has been one of the fungal species that share the interest of investigators in the field of immune modulation, they have demonstrated that immunization with mannan (a mannoproteins fraction) and mannoproteins derived from digested cell walls of *C. albicans* induced resistance to a systemic candidiasis (3 and 4). The plant extracts derivatives or their products have also been the interest of investigators as immunomodulators to overcome the disadvantage of biological and chemical immunomodulators. One of these plants is *Azadirachta indica*, which is more popular with the name Neem, and has the advantage to be a medicinal plant with a wide range of applications in folkloric medicine (5). Furthermore recent investigations demonstrated several biological and pharmaceutical potentials; for instance, anti-viral, anti-bacterial, anti-parasitic, anti-cancer and immune stimulant properties of the Neem (5, 6, 7 and 8). Prevention of brucellosis in human still depends on the eradication or control of the disease in animal hosts. The exercise of hygienic precautions to limit exposure to infection through occupational activities, and the effective heating of dairy

products and other potentially contaminated foods, but vaccination may have important role in the prevention of human disease (9). Various vaccine preparations have been employed; including live attenuated *B. abortus* strains 19-BA and 104M (used mainly in the former Soviet Union and China) (10). Other authors also (11) noted that peptidoglycan of the *B. abortus* cell wall produced a good protection in mice against brucellosis. Till now the immunologists are engaged to design vaccines and vaccine strategies to maximize the activation of the immune system and to avoid the unfavorable complications resulted from *Brucella* vaccines. One of these strategies is the employment of immune modulators to potentiate the immune response and increase the effectiveness of vaccines.

MATERIALS AND METHODS

All experiments was done in the National centre of Drug control and Research at a period extended from June/2007 to June/2009 on male and female albino mice (Blab-c), supplied by this Centre. Their age range at the start of experiments 6-8 weeks. They were housed in bio-clean hoods at 20-25ºC with light: dark periods of 14:10 hours. They had free access (*ad libitum*) to food (standard pellets) and water, and their average weight was 22 ± 3 grams at the start of experiments. Before carrying out the experiments, the mice were left in separate cages for one week to experience the acclimatization period. The study was conducted on two main groups (160 mice for each group), Each group was divided into eight subgroups 20 mice each subgroups (Subgroup I, treated with distilled water, II with the *Brucella* Rev-1 vaccine, III with mannoproteins, IV with neem aqueous extract, V with ethanolic neem extract, VI, VII and VIII: with mannoproteins, neem aqueous extract and neem ethanolic extract respectively. Then they were vaccinated with *Brucella* Rev-1). All these treatments were carried out on day 1 and then vaccinated with brucella Rev-1 vaccine on day 4 .Then the mice were tested as follows , on day 8 after vaccination(for investigated of (peripheral blood lymphocyte MI index%) and(NBT index%), day 14 for (Skin test).Two extracts (aqueous and ethanolic) were prepared. The aqueous and ethanolic extract preparation was according to a method presented by (3). The Mannoproteins were prepared from the cell wall of a *Candida albicans* isolate. The isolate, which was obtained from the vaginal swab of a healthy woman, was supplied by the Central Health Laboratory (Iraq/Baghdad). The *C. albicans* sample was maintained on yeast extract peptone glucose agar supplemented with amino acids (12) .The cell wall mannoproteins of *C. albicans* were prepared according to a method presented by (12). The doses of both plant extracts and mannoproteins represented 10% of the calculated LD_{50} (neem extracts: 3.8096 g/Kg mannoproteins: 5.7144 mg/Kg), which were given subcutaneously by Dixon (14). Mice of the second main group were injected with the immune suppressive drug prednisolone (5mg/Kg) 5 days prior to the treatments (14). The dried lyophilized seed of *Brucella Mulitensis Rev-1* strain was supplied by the Central Veterinarian Laboratory Iraq/Baghdad, and this laboratory received the strain from the Food and Agriculture Organization (FAO). Brucelline was prepared according to a method presented by (9).The protein concentration was adjusted to 46 µg/ml (11). (NBT Index) was carried out according to a method presented by (12) for all the mice.

$$\text{NBT Index}(\%) = \left(\frac{\text{Number of Positive Nutrophils}}{\text{Total Number of Neutrophils}}\right) \times 100$$

The procedure of (15) was followed to assess the mitotic activity of lymphocytes after an *in vitro* stimulation with PHA and brucelline.

$$\text{MI Index}(\%) = \left(\frac{\text{Number of Mitotic Cells}}{\text{Total Number of cells}}\right) \times 100$$

Statistical Analysis

The values of the investigated parameters were given in terms of means ± standard errors (S.E.), and differences between means were assessed by analysis of variance (ANOVA), least significant difference (LSD) and Duncan test, using the computer programmer SPSS (Statistical Package of Social Sciences) version 7.5. The difference was considered significant when the probability value was equal or less than 0.05. A further estimation was also given; it was treated efficiently (Perez-Serrano et al., 1997), which was calculated according to the following equation:

$$\text{Treatment efficiency}(\%) = \left(\frac{A - B}{B}\right) \times 100$$

A = Treated groups; B = Negative control group.

RESULT AND DISCUSSION

The results of NBT index were given in (table 1), while the treatment efficiency for each group of treated mice was presented in (fig.1). All mice without prednisolone treatment showed different significant increases ($P \leq 0.05$, $P \leq 0.01$) in the NBT index as compared to (control group). The best treatment efficiency was recorded in group VII (39%). A similar outcome was demonstrated (a significant increase ($P \leq 0.05$, $P \leq 0.01$) in the NBT index) in mice with prednisolone treatment, but the highest treatment efficiency was recorded in group VIII (30%). Furthermore, all mice without prednisolone treatment showed a significant increase ($P \leq 0.05$ $P \leq 0.01$) in the NBT index as compared to mice with prednisolone treatment.

TABLE 1: Nitro blue tetrazolium (NBT) index in mice groups

Groups	NBT index (mean ± S.E., %)*		P-value ≤
	Without Prednisolone Treatment	With Prednisolone Treatment	
I	8.00 ± 0.58 [e]	6.00± 1.15 [c]	0.05
II	21.00 ± 1.15 [c]	17.00 ± 0.58 [ab]	0.01
III	19.00 ± 0.58 [cd]	14.33 ± 0.88 [b]	0.01
IV	21.00 ± 0.58 [c]	18.67 ± 1.45 [ab]	0.01
V	16.00 ± 0.58 [d]	14.00 ± 1.15 [b]	0.05
VI	18.00 ± 1.15 [cd]	12.00 ± 1.15 [bc]	0.01
VII	39.00 ± 0.58 [a]	23.33 ± 4.7 [a]	0.01
VIII	30.67 ± 1.8 [b]	24.00 ± 3.05 [a]	0.01

*Different letters: Significant difference (P≤0.05) between means of the same column.
**The comparison is between means of the two columns (horizontal comparison).

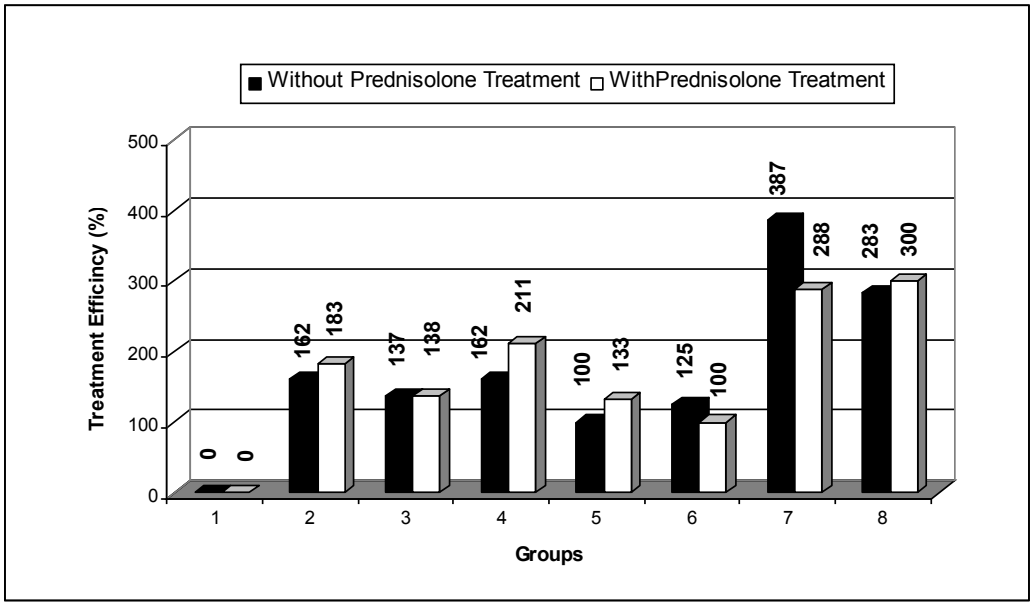

FIGURE 1: Treatment efficiency of NBT index in mice groups

Results of NBT index, which showed a significantly increased percentage in treated mice, are also in favour of such agreement, NBT reduction by polymorph nuclear cells may require oxidative metabolism by the hexose monophophate shunt, and is impermeable to cell membrane, but it enters the cell during the process of Phagocytosis, and it is reduced by diaphoreses activity within phagosome (3 and 16). Attachment of the phagocyte to the organism is an important interaction, which may determine whether the uptake is subsequently occurred or not, and whether the killing mechanisms are triggered or not (17). They can bind to B-glucans and to the lipopolysaccharide endotoxin of Gram-negative bacteria and this can lead to complement deposition via the alternative or classical pathways (18). Although macrophages and Monocytes possess killing mechanisms in the resting state, these mechanisms can be enhanced, and new mechanisms can be expressed when they are activated. Activation can occur through exposure to microbial products (i.e. *C. albicans* cell wall mannoproteins and brucella vaccine) and/or materials extracted from plants (*i.e.* ethanolic and aqueous extracts of neem seeds). Such picture is enhanced by the findings of the present study and also confirmed by other investigators (5 and 8). Such immunomodulators can cause a direct activation of phagocytes, or indirect activation through triggering cytokine release from them. Once the organism is internalized, it is exposed to an array of killing mechanisms, oxygen-dependent killing mechanisms and reactive nitrogen intermediates (RNI) (19 and 20). For optimal expression of this mechanism, macrophages need both activation by IFN-γ and triggering by TNF-alpha, and this mechanism enables murine macrophages to kill mycobacterium and probably other intracellular bacteria like *Brucella* (21). The results of lymphocyte transformation index were given in table (2), while the treatment efficiency for each group of treated mice was presented in figure (2). In mice without prednisolone treatment, the highest value of lymphocyte transformation index was recorded in group VII (20%), followed by groups VI and VIII (16.43 and 15.60%, respectively). These values were significantly higher than the corresponding value of group I (4.30%), and associated with treatment efficiencies of 365, 282 and 262%, respectively. The rest of groups also showed a significant increased index (P≤0.05, P≤0.01), but with a lesser extent as compared to group I. In mice with prednisolone treatment, similar results were obtained and

groups VI, VII and VIII recorded a significant increased index of lymphocyte transformation($P \leq 0.05$ $P \leq 0.01$) as compared to the corresponding value of group I (13.33, 16.67 and 13.57%, respectively *vs*. 3.60%). When mice without prednisolone treatment compared to mice with prednisolone treatment, all groups in the latter mice showed a significant decreased index of lymphocyte transformation.

TABLE 2: Lymphocyte transformation index in mice groups

Groups	Lymphocyte Transformation Index (mean ± S.E.; %)*		P-value ≤
	Without Prednisolone Treatment	With Prednisolone Treatment	
I	4.30 ± 0.06 [e]	3.60 ± 0.23 [e]	0.05
II	12.83 ± 1.17 [c]	11.17 ± 0.44 [c]	0.05
III	8.53 ± 0.29 [d]	7.87 ± 0.09 [d]	0.05
IV	8.00 ± 0.58 [d]	7.13 ± 0.52 [d]	0.05
V	7.67 ± 0.35 [d]	6.83 ± 0.22 [d]	0.05
VI	16.43 ± 0.38 [b]	13.33 ± 0.88 [b]	0.01
VII	20.00 ± 0.29 [a]	16.67 ± 0.33 [a]	0.01
VIII	15.60 ± 0.35 [b]	13.57 ± 0.47 [b]	0.01

*Different letters: Significant difference ($P \leq 0.05$) between means of the same column

**The comparison is between means of the two columns (horizontal comparison)

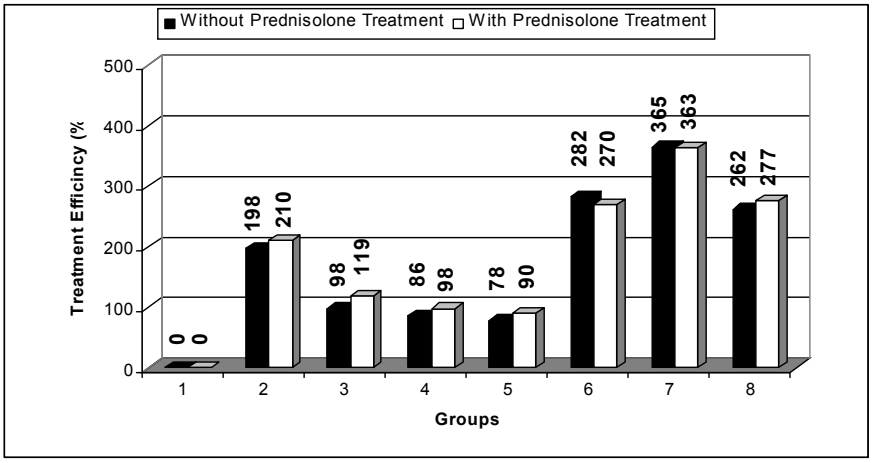

FIGURE 2: Treatment efficiency of lymphocyte transformation index in mice groups.

The results of DTH index were given in table (3), while the treatment efficiency for each group of treated mice after 24 and 48 hours is presented in figures (2) and (3). In mice without prednisolone treatment, measuring the DTH after 24 hours revealed that all groups (with the exception of group V; 0.64 mm) showed an increased DTH index, the differences were significant($P \leq 0.05$, $P \leq 0.01$) with the exception of group IV, in which the different was not significant as compared to group I (1 mm), and almost a similar picture was observed after 48 hours, but the index values were lower and group VIII showed a non-significant decreased DTH index as compared to the corresponding value in group I (0.83*vs*. 0.9 mm).

TABLE 3: Delayed type hypersensitivity (DTH) index in mice groups

Groups	DTH index (mean ± S.E., mm)*				P- value ≤	
	Without Prednisolone Treatment		With Prednisolone Treatment			
	24 Hours	48 Hours	24 Hours	48 Hours	I	II
I	1.0 ±0.12[c]	0.90±0.06[cd]	0.67±0.04[e]	0.50±0.01[d]	N.S.	0.01
II	1.43±0.02 [b]	1.23±0.15[b]	0.75±0.08[de]	0.77±0.04[c]	0.05	0.05
III	1.40±0.11[b]	1.27±0.12[b]	1.29±0.1[b]	1.12±0.04[b]	0.01	N.S.
IV	1.19±0.03 [c]	0.98±0.06[bc]	0.96±0.02[cd]	0.91±0.03[c]	0.05	N.S.
V	0.64±0.04[d]	0.50±0.01[e]	0.57±0.04[e]	0.41±0.01[b]	0.05	0.05
VI	3.08±0.07[a]	2.69±0.02[a]	1.69±0.07[b]	1.58±0.06[d]	0.01	0.01
VII	1.66±0.05[b]	0.83±0.12 [c]	1.04±0.16[c]	0.49±0.06[d]	0.05	0.01
VIII	1.65± 0.04[b]	0.77±0.03[cd]	1.03±0.06[cd]	0.87±0.08[c]	0.01	0.05

*Different letters: Significant difference ($P \leq 0.05$) between means of the same column.

**The comparison is between means (I: 24 hours; II: 48 hours) of the two columns (horizontal comparison).

In mice with prednisolone treatment showed a similar outcome was observed, and index was significantly increased in groups II (48 hours; 0.77 mm), III (24 hours: 1.29; 48 hours: 1.02 mm), IV (24 hour: 0.96; 48 hours: 0.91 mm), VI (24 hours: 1.69; 48 hours: 1.58 mm), VII (24 hours: 104 mm) and VIII (24 hour: (24 hour: 1.03; 48 hours: 0.87 mm); 48 hours: 0.91 mm) as compared to the corresponding values in group I (24 hours: 0.67 mm; 48 hours: 0.5 mm), but the DTH values were significantly lower than the corresponding values in mice without prednisolone treatment, with the exception of group I (24 hours), group IV (48 hours), in which the differences were not significant. The highest treatment efficiency after 24 hours was recorded in group VI (200%), while after 48 hours, it was also in group VI, but with prednisolone treatment (229%).

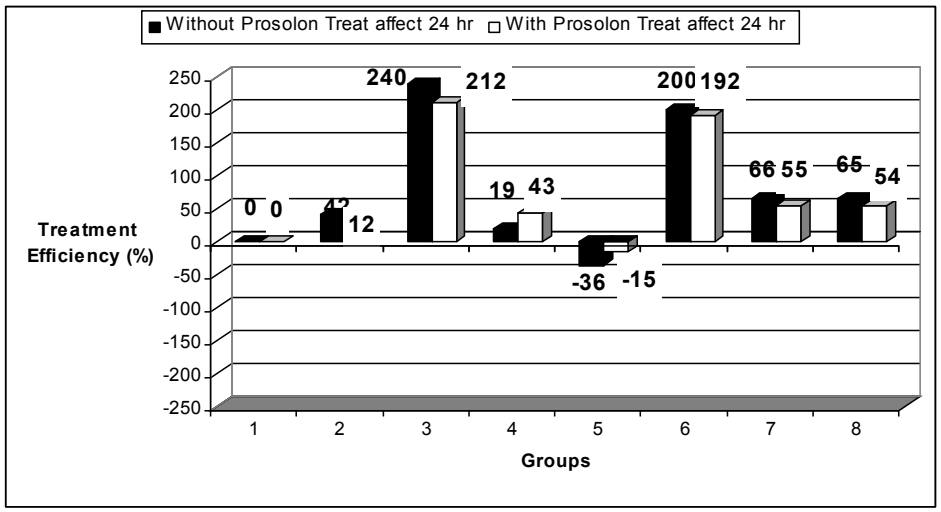

FIGURE 3: Treatment efficiency of delayed-type hypersensitivity index after 24 hours in mice groups.

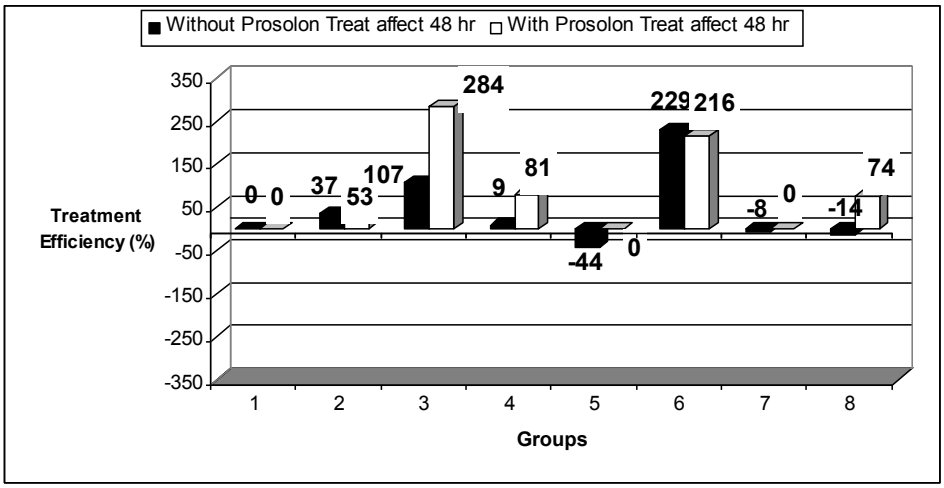

FIGURE 4: Treatment efficiency of delayed-type hypersensitivity index after 48 hours in mice groups.

Both immunomodulators were assessed for their effectiveness on cell-mediated immunity, which can be assessed by determining the level of delayed-type hypersensitivity (DTH) response, and the latter is an important host defense mechanism against brucellosis. The brucelline antigen preparation used for the detection of DTH is a heterogeneous mixture of relatively high-molecular weight (more than 50000 Daltons) polysaccharides and proteoglycans, which can elicit specific immune response against *Brucella* Rev-1 strain (21). In this regard, mice treated with 14.286 µg/mouse of *C. albicans* mannoproteins prior to the time of vaccination produced a significant DTH response against brucelline in comparison to the corresponding control, and the highest thickness was produced after 24 hours of brucelline injection, especially in those mice that were treated with prednisolone. Such findings suggest the importance of these materials as immunomodulators in a combination with a *Brucella* vaccine programmed. These results came to confirm the findings of (2,17,18 and (22). These investigators collectively agreed that the mannoproteins of *C. albicans* cell wall are the primary component recognized in the cell mediated immune response, and mannoproteins have a variety of non-specific immunomodulators that can effect and modulate the immune response through inducing lymphocyte proliferation and Neutrophils activation with the consequent production of cytokines that can modulate the immune response against brucelline antigen. In this regard, DTH reactions develop when antigen activates sensitized T_{DTH} cells, and these cells generally appear to be a T_H1 subpopulation although T cytotoxic may also be involved.

An activation of T_{DTH} cells results in secretion of various cytokines including IL-2, IFN-γ, macrophage-inhibiting factor and tumor necrosis factor (23). The overall effect of these cytokines is to draw macrophages to the area of injection and activated them, promoting increased phagocytic activity and increased concentrations of lytic enzymes for more active killing. As lytic enzymes leak out of the activated macrophages into the surrounding tissue, localized tissue destruction can occur. These reactions typically take 48-72 hours to develop, which is the time required for initial T_{DTH} cell activation and cytokine secretion to mediate the accumulation of macrophages and the subsequent release of their lytic enzymes(24).

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