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Author(s)	OKUYAMA, Harue; TAKEDA, Junko; ONOÉ, Kazunori et al.
Citation	北海道大学免疫科学研究所紀要, 38, 13-23
Issue Date	1978-03
Doc URL	<a href="https://hdl.handle.net/2115/26504">https://hdl.handle.net/2115/26504</a>
Type	departmental bulletin paper
File Information	38_P13-23.pdf



## Histological Studies on Adjuvanticity of BCG-Cell Walls

— Comparison of adjuvanticity between oil-in-water  
and water-in-oil forms\* —

Harue OKUYAMA, Junko TAKEDA, Kazunori ONOÉ  
and Kazuo MORIKAWA

*Division of Pathology, Institute of Immunological Science,  
Hokkaido University, Sapporo, Japan*

(Received for publication, December 1, 1977)

**Abstract** Adjuvant activity of BCG whole cell walls in rabbits was evaluated in bovine gamma globulin immunization using two different adjuvant forms, oil-in-water and water-in-oil, from the histological viewpoint.

Cell walls enhanced strongly both circulating antibody formation and delayed-type hypersensitivity. On the other hand, cell walls in the o/w form augmented delayed-type hypersensitivity alone, with antibody formation to a lesser degree than was seen in the w/o group. On histological examination, the injected sites and regional lymph nodes exhibited remarkable proliferative responses.

Differences in the histological changes induced by both adjuvant forms were found in quantity and duration rather than in quality. In the o/w groups the maximum response was reached early and declined thereafter, whereas the w/o groups attained the maximum slowly and maintained a high response for a long time.

Immunofluorescently, the appearance, differentiation, and numbers of antibody-forming cells were in parallel with the histological changes and antigen retention.

Furthermore, the o/w form of adjuvant evoked a high delayed-type hypersensitivity, similar to the w/o form, but provoked a scanty serum antibody formation and also induced a comparatively weak histological change in the injected sites and in the regional lymph nodes.

Since the presentation by Freund<sup>0)</sup> killed tubercle bacilli in the form of water-in-oil has been used as the most popular adjuvant materials. Its purpose is heighten circulating antibody formation. Among mycobacterial components fractionated, BCG-cell walls were reported to induce high resistance against virulent infection<sup>1,10)</sup> and an enhanced delayed type hypersensitivity, when used in oil-in-water form, attached to a small amount of Drakeol, a mineral oil. However this form is reported to be not as effective in antibody formation as the water-in-oil form.<sup>3,4)</sup>

We can expect a different mode of action of BCG-cell walls in the adjuvant effect, when different adjuvant forms are used. The purpose of the present study is to clarify the mechanism that causes these differences by the use of oil-in-water and water-in-oil forms of adjuvant. We have undertaken some experiments using BCG-cell walls and cell wall skeletons as adjuvant in the case of immunization with sheep red blood cells. The present paper demonstrates the results of experiments using bovine gamma globulin and BCG-cell walls.

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\* This work was supported by a grant-in-aid for scientific research from the Ministry of Education, Science and Culture of Japan (010714/1975 & 112118/1976) and by U.S.-Japan Cooperative Medical Science Program from the Ministry of Health and Welfare of Japan.

## MATERIALS AND METHODS

**Animals.** White rabbits weighing 3 to 4 kg were used.

**Antigen.** Bovine  $\gamma$ -globulin (BGG) was used (Gamma globulin Cohn Fraction II Bovine, lot No. 1641, ICN Pharm. Inc.)

**Adjuvant.** BCG-cell walls (CW) fractionated in Rocky Mountain Laboratory, NIH, were used. Immunogens containing BGG and CW were prepared in the following two forms: 1) The oil-in-water (o/w) form. Five mg of BGG and 0.5 mg of CW were emulsified together with a small amount of Drakeol 6-VR and then diluted with 0.2% Tween 80 in saline to a concentration of 5 mg BGG and 0.5 mg CW in 0.5 ml. 2) The water-in-oil (w/o) form. Freund's incomplete adjuvant (FIA) containing CW was emulsified with an equal volume of BGG saline solution to a final concentration of the same as in the o/w form.

**Immunization.** Each group of 12 rabbits received an injection of 0.5 ml immunogens into each hind footpad. Control groups were injected with BGG saline solution or o/w or w/o form of BGG without CW in the same amount as in the experimental groups. Each group was inoculated with the following form of immunogens:

Group 1 (G-1): BGG in saline

Group 2 (G-2): BGG in o/w form adjuvant without CW

Group 3 (G-3): BGG in w/o form adjuvant without CW

Group 4 (G-4): BGG with CW in o/w form adjuvant

Group 5 (G-5): BGG with CW in w/o form adjuvant

At 3 hours, 1, 6, 10 and 19 days after immunization the animals were tested as follows:

**Hemagglutinin titer.** Sera were obtained from each animal and the antibody titer against BGG was determined by passive hemagglutination.

**Weight of the regional lymph nodes.** The popliteal lymph nodes from 4 animals at given days in each group were removed and weighed after trimming adipose tissues surrounding the nodes.

**Histological examination.** The popliteal lymph nodes and injected sites of both footpads were removed. A half portion of each tissue was fixed with Carnoy's fixative and paraffin sections were stained with hematoxylin-eosin or methylgreen-pyronin. The other portion was fixed quickly with 95% ethanol by continuous stirring in a cold room and paraffin sections were made for immunofluorescent staining. The sections were stained for BGG antigen by the direct method and for anti-BGG antibody by the sandwich method using rabbit anti-BGG globulin labeled with fluorescent isothiocyanate.

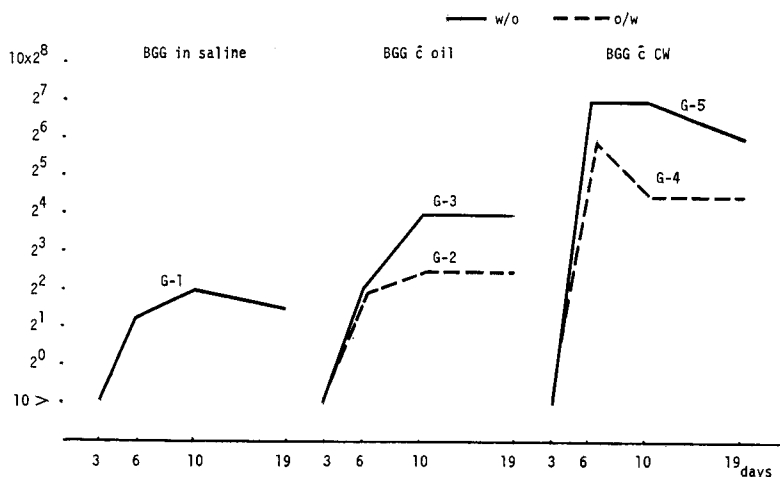
**Skin test.** Skin test was performed on day 4, 8 and 17 after immunization by intradermal injection of 0.1 ml of 0.1% BGG saline solution. The reaction was judged by measuring the size of redness and thickness of indurated skin at 6, 24 and 48 hours after injection.

## RESULTS

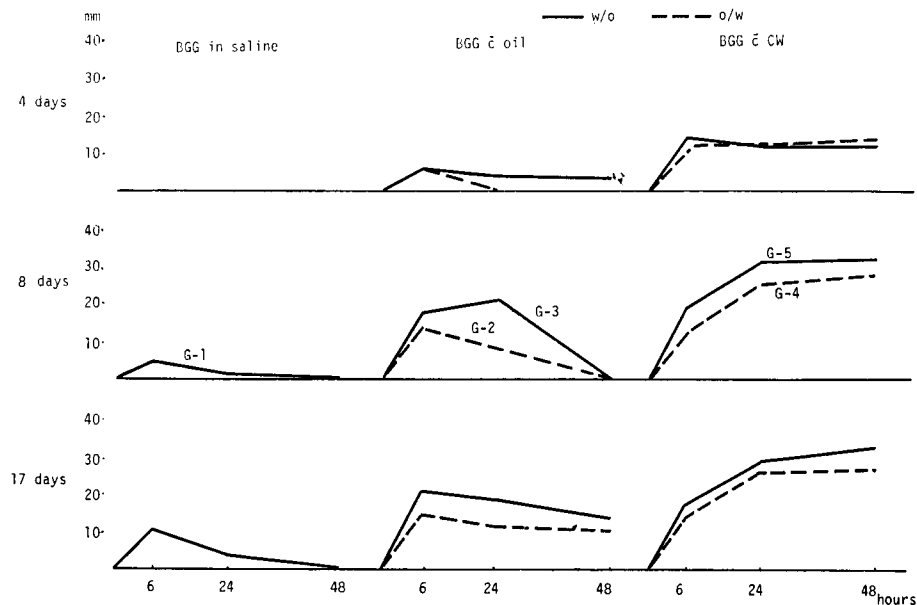
### Serum antibody titer:

Group 4 animals, oil-in-water CW adjuvant group, showed their maximum titer on day 6 and then the titer decreased, while group 5, water-in-oil group presented a high level titer and maintained this longer. (Fig. 1)

### Skin reactions:



**Fig. 1.** Hemagglutinin Titer of Serum from Rabbits Immunized with BGG and BCG-CW



**Fig. 2.** Skin Reaction in Rabbits Immunized with BGG and BCG-CW (Erythema)

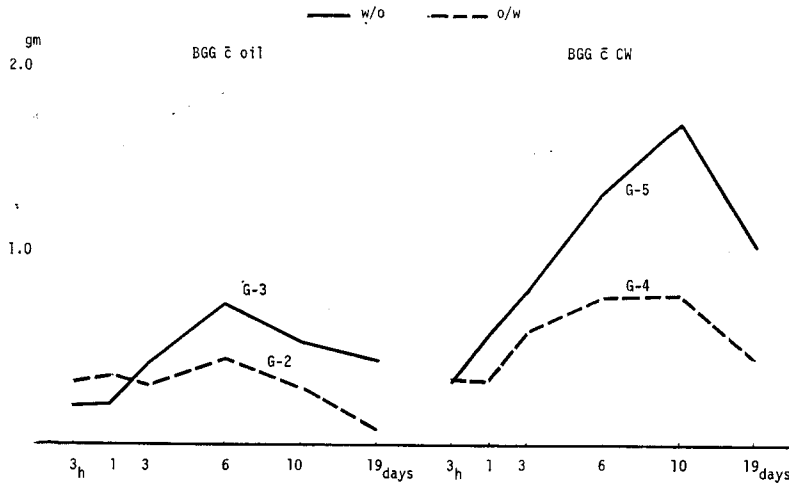
The time course of the reaction is described in Fig. 2. Oily adjuvant, as seen in G-2 and G-3, intensified the reactivity slightly, but the reactions were of immediate type. Addition of CW, as seen in G-4 and G-5, enhanced remarkably, but converted the reaction to that of delayed type, and showed 48 hours' peak. There was no difference in the intensity of reactions between G-4 and G-5. G-4 and G-5 animals also developed tuberculin hypersensitivity. The tuberculin reactivity in G-4 animals appeared earlier and was more intense than in G-5.

#### **Weight of the regional lymph nodes :**

The popliteal lymph nodes in G-5 showed the most drastic increase in weight, reaching a peak on day 10 (Fig. 3). G-4 showed only slight increase, comparable to G-3.

**Histological changes :**

**Regional lymph nodes :** Addition of a small amount of Drakeol or FIA (G-2 and G-3) to BGG antigen enhanced a development of germinal center and an enlargement of the paracortical



**Fig. 3.** Weight of Regional Lymph Node from Rabbit Immunized with BGG and BCG-CW

**Table 1.** Histological Changes in Regional Lymph Nodes from Rabbits Immunized with BGG and BCG-Cell Walls

After immun.	Germinal centers					Pyroninophylic cells in									
	G-1*	G-2	G-3	G-4	G-5	Cortico-medull. junction					Medullary cord				
	G-1*	G-2	G-3	G-4	G-5	G-1	G-2	G-3	G-4	G-5	G-1	G-2	G-3	G-4	G-5
3 h.	-	±	±	-	-	+	+	±	±	±	±	±	±	-	±
1 d.	-	+	+	+	-	+	+	-	-	-	+	+	±	+	+
3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
19	+	+	±	+	+	±	±	-	+	+	±	±	±	+	+

After immun.	Enlargement of paracortex					Pyroninophylic cells in paracortex					Epithelioid cells				
	G-1*	G-2	G-3	G-4	G-5	G-1	G-2	G-3	G-4	G-5	G-1	G-2	G-3	G-4	G-5
3 h.	-	±	±	-	-	-	-	±	±	±	-	-	-	-	-
1 d.	±	+	-	±	+	+	±	+	+	±	-	-	-	-	-
3	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
6	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+
10	-	+	+	+	+	-	+	+	+	+	-	-	±	+	+
19	-	-	±	+	+	-	-	±	+	+	-	-	-	+	+

\* G-1: BGG in saline, G-2: BGG in o/w form, G-3: BGG in w/o form, G-4: BGG with CW in o/w form, G-5: BGG with CW in w/o form

area. These changes were stronger in G-3 than G-2 (Table 1). When CW was given with these immunogens, the regional lymph nodes showed further severe changes such as large pyroninophilic cell proliferation in the enlarged paracortical area, plasma cell proliferation in the cortico-medullary junction and medullary cord, and the epithelioid cell granuloma in the cortex. While these changes in o/w group, G-4, appeared early and then declined after the maximum of day 6, w/o group, G-5, showed a little later appearance, the maximum from day 6 to 10, and maintained the strong changes until day 19. The epithelioid granuloma developed on day 6 and occupied about a half part of the node on day 10 in o/w group and on day 19 in w/o group.

**Footpads:** G-1 animals showed an infiltration of a large number of polymorphonuclear cells in the injected sites on day 1 and a decrease in number of infiltrating cells thereafter. Animals of G-2 and G-3 enhanced the polymorphonuclear infiltration. W/o group showed a prolonged polymorphonuclear infiltration and a small abscess formation. Some infiltrating macrophages showed ingestion of the oil droplets. Both o/w and w/o groups produced granulation tissues around the polymorphonuclear foci after day 3. On day 19, while o/w group showed only small granulomatous foci, w/o group developed large granulomata consisting mainly of macrophages.

Addition of CW greatly enhanced the polymorphonuclear infiltration and abscess formation after day 3, especially in w/o group. Granuloma of macrophages and fibroblasts around the abscess on day 3 transformed to the epithelioid cell granuloma after day 10. A large number of plasma cells was seen around small vessels. In the granuloma, some cystic spaces, probably showing a storage of oil droplets, were found till the later stage. The size of granuloma was significantly large in w/o group than in o/w group.

#### Antigen trapping and antibody formation in the injected sites and regional lymph nodes:

In G-1 animals a small amount of antigen could be seen by immunofluorescent-antibody staining in the injected sites and along the sinus linings of the regional lymph nodes only for the first 3 days after immunization (Table 2). Many antibody-forming cells appeared on day 6 in the medullary cords of the nodes and disappeared rapidly thereafter. No antibody-forming cells were detected in the injected sites throughout the experiment.

In G-2 and G-3, antigens were detected in the nodes and injected sites in a small quantity. Antibody-forming cells were found in the cortico-medullary junctions and medullary cords of the

**Table 2.** Immunofluorescent Findings of Regional Lymph Nodes and Injected Sites in Rabbits Immunized with BGG and BCG-CW

After immun.	G-1		G-2		G-3		G-4		G-5	
	LN*	FP**	LN	FP	LN	FP	LN	FP	LN	FP
	Ag**Ab	Ag Ab	Ag Ab	Ag Ab	Ag Ab	Ag Ab	Ag Ab	Ag Ab	Ag Ab	Ag Ab
3 h.	± -	NT**NT	+ -	NT NT	# -	NT NT	# -	NT NT	+ -	NT NT
1 d.	± -	± -	+ -	± -	+ -	+ -	# -	# -	# -	# -
3	± -	- -	± -	± -	+ -	+ -	# -	# -	# -	# -
6	- #	- -	- #	- #	+ #	+ +	+ #	+ #	+ #	+ #
10	- +	- -	- #	- ±	± #	+ #	± #	+ #	+ #	+ #
19	- -	- -	- ±	- -	± +	+ +	- +	± +	+ #	+ #

\*LN: Regional lymph node, \*\*FP: Footpad injected site, \*\*Ag: BGG antigen, Ab: Antibody-forming cell, \*\*NT: Not tested

nodes and in the perivascular areas of the injected sites on and after day 6. Both antigen-containing and antibody-forming cells were demonstrated for a long period in w/o group, though in a small quantity.

Addition of CW to both o/w and w/o form of antigen (G-4 and G-5) enhanced a retention of antigen remarkably at the injected sites such as in the center of abscess foci in the early stage and at the margin of cystic spaces of injected oil in the later stage. In the lymph nodes antigens were detected along the lining of peripheral, medullary and cortical sinuses and also in cytoplasm of macrophages in the sinuses. These antigens were retained more and longer in w/o group than in o/w group. The antibody-forming cells were found in the perivascular area of injected sites and mainly in the cortico-medullary junctions and medullary cords of the lymph nodes, but in a small number in the cortical area. These cells were detected on and after day 6, which decreased in number in the later stage in o/w group while they were continuously demonstrated in a large number until day 19 in w/o group.

### DISCUSSION

Ribi et al.<sup>7,8)</sup> fractionated cell walls (CW) from *Mycobacteria*. Animals receiving an intravenous injection of the CW associated a small amount of Drakeol acquired a high level of immunity against infection of virulent tubercle bacilli. Furthermore CW gave an evidence for a strong adjuvant activity on antibody formation, induction of delayed hypersensitivity in sensitization with foreign proteins or heterologous red blood cells and induction of a high level of antitumor immunity as well.<sup>2,8,9,13,15)</sup> In most experiments the cell walls were used in the oil attached form (o/w). This type of immunogen markedly induced increased cell-mediated immunity but slight enhancement of circulating antibody formation. The o/w form of CW adjuvant appeared to be the most suitable form for induction of cell-mediated immunity.<sup>3)</sup> On the other hand, CW with FIA (w/o) enhanced markedly both circulating antibody level and cell-mediated immunity.<sup>4)</sup> In the previous study<sup>10)</sup> o/w form of CW induced a remarkable delayed hypersensitivity comparable to that in w/o form, but lower antibody formation. We attempted to clarify the mechanisms causing the differences in immune responses resulted from the use of different adjuvant form.

Animals which received antigens containing CW showed a marked enlargement of the paracortical area accompanied by prominent proliferation of large pyroninophilic cells, generation of those cells in the cortico-medullary junctions and expansion of the medullary cords, and epithelioid cell granuloma formation. All these changes were greater in the CW groups than in the control groups. The histological differences in o/w or w/o groups were found in the time of appearance and the duration of the changes. Animals injected with o/w form of antigen indicated an early appearance of changes showing the maximum on day 3 to 6 and a decline thereafter, while those injected with w/o form showed the maximum on day 6 to 10 and the striking changes continued during the whole experimental period. An early transient but prominent proliferation of plasma cells followed by the decline in the regional lymph nodes and injected sites of o/w group, and the maintenance of intensive plasma cell proliferation in w/o group were well correlated with the circulating antibody level. On the other hand, delayed hypersensitivity, regardless of the differences in the form of adjuvant, was similarly intensive in both groups, and the intensity was parallel with the degree of epithelioid cell granuloma formation in the regional lymph nodes and the injected sites. Whether the granuloma cells are themselves effector cells or producers of a factor or factors for cell-mediated immunity, or only present a histological expression of enhanced cell-mediated immunity or not is not clear. However, the fact that the enlargement of the paracortical area

and the proliferation of large pyroninophilic cells in these areas were severer and lasted longer in w/o group than in o/w group suggests that these changes not only participate in cell-mediated immunity but play a part in enhancement of antibody level as helper cell proliferation.

The relationship between the long lasting reaction and the existence of antigen in the injected sites and regional lymph nodes of w/o group was examined by the immunofluorescent method. A significant amount of antigen was specifically stained at the margin of cystic spaces in the injected sites and on the sinus walls in the cortex of regional nodes during all the experimental period. Champlin and Hunter<sup>9)</sup> described that the effective adjuvant can localize and maintain antigen in the paracortical area of the regional lymph nodes and keep the antigen in the injected sites.<sup>12)</sup> It might be considered that the presence of antigen during a long period means a continuous supply of appropriate antigenic stimuli for the plasma cell proliferation. Indeed, the existence of antigen in the injected sites and the regional lymph nodes was accompanied by the appearance of a number of specific antibody-forming cells therein. Westwater<sup>14)</sup> reported that antibody production in the injected sites played an important role as well as that in the regional lymph nodes.

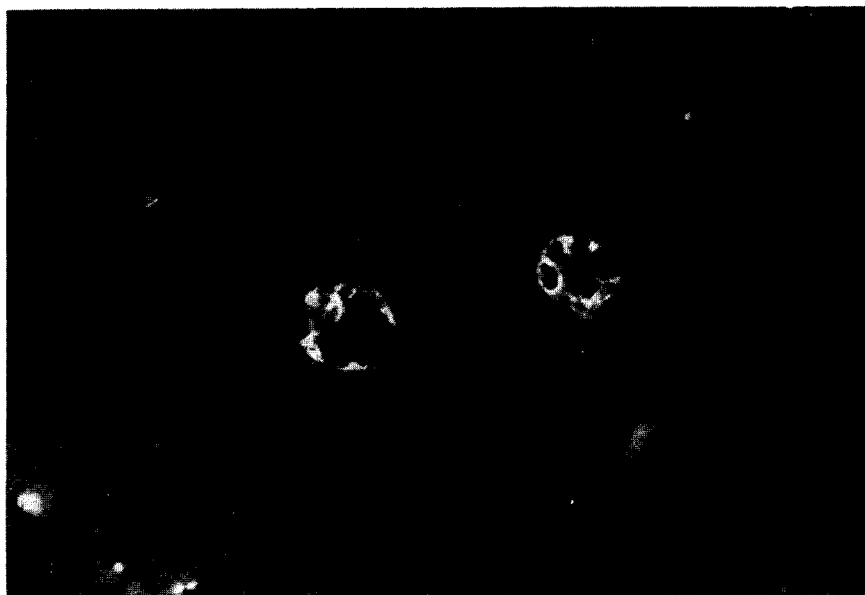
These results suggest the requirement of continuous presence of antigen in a considerable amount for enhanced antibody formation in the regional lymph nodes and injected sites. On the other hand, the enhanced and long lasting cell-mediated immunity could be produced without the presence of antigen, if once induced to an appropriate level. This may be due to the difference in the lifespan between B-cells engaging in antibody formation and T-cells, effector cells, for cell-mediated immunity.

In brief, o/w form of adjuvant evoked a high delayed-type hypersensitivity, similar to the w/o form, but provoked a scanty serum antibody formation and also induced a comparatively weak histological change in the injected sites and in the regional lymph nodes. From these results and previous work<sup>10)</sup> using cell walls and PPD as test antigen, the o/w form of BCG cell wall immunization appears to be the preferred method for clinical application of immunotherapy.

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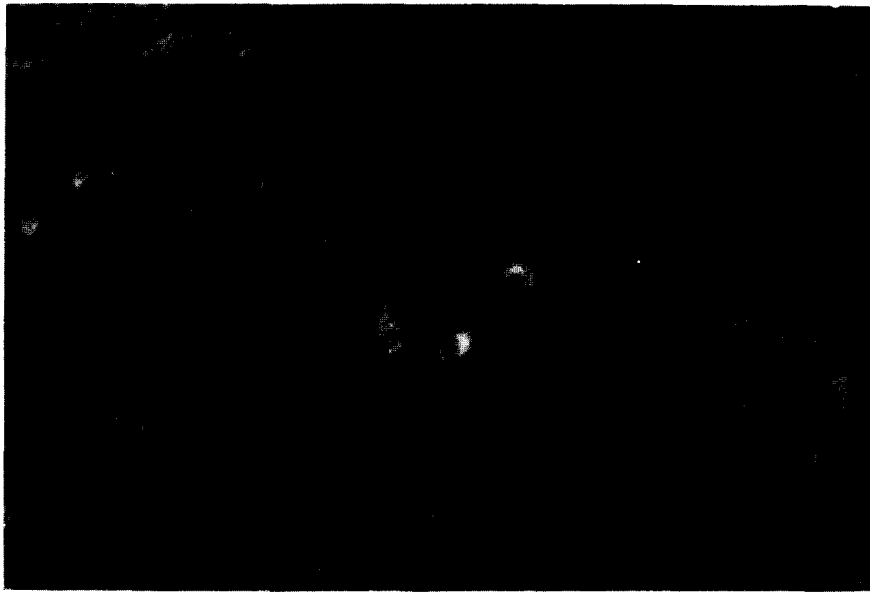
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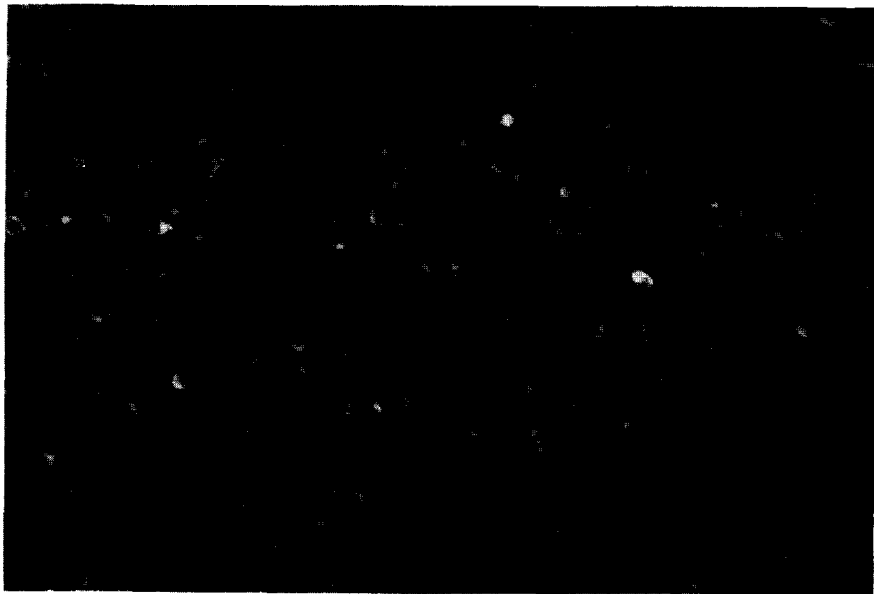
**Photo. 1** Group 4 animal. (BGG with CW adjuvant in the form of o/w)  
BGG antigens are stained at the margin of oil droplets in the footpad. Three days after sensitization. Direct immunofluorescent method.  $\times 250$



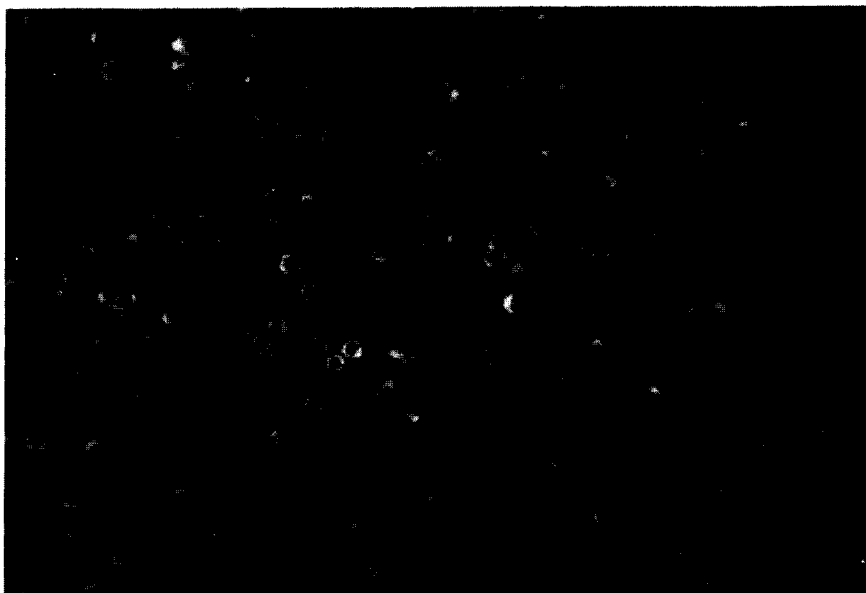
**Photo. 2** Group 5 (BGG with CW adjuvant in the form of w/o)  
BGG antigens are found at the margin and around the large oil spaces in the footpad. Nineteen days after sensitization. Direct immunofluorescent method.  $\times 250$



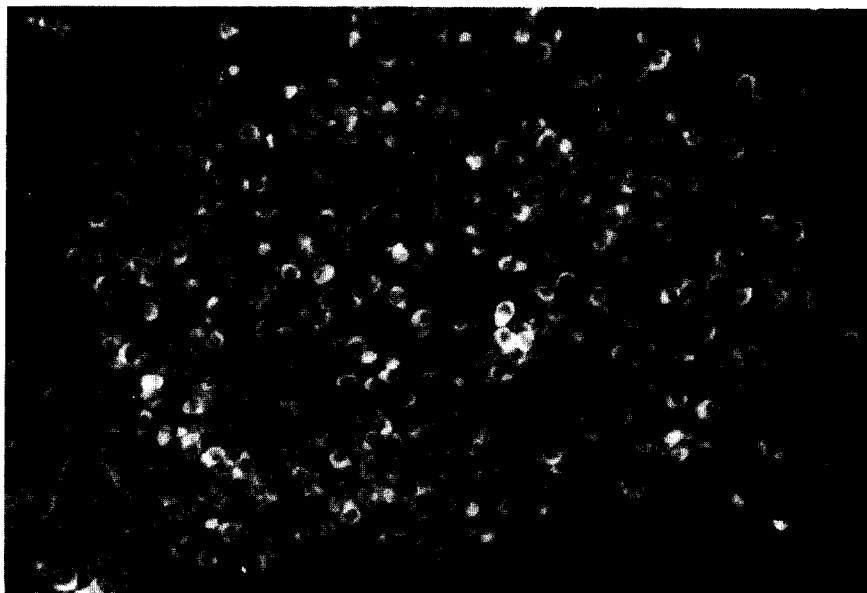
**Photo. 3** Group 4. A few antibody-forming cells are seen in the perivascular area of the footpad. Nineteen days after sensitization. Sandwich immunofluorescent method.  $\times 400$



**Photo. 4** Group 5. Many antibody-forming cells are found in granulation tissues around epithelioid cell granuloma in the footpad. Nineteen days after sensitization.  $\times 250$



**Photo. 5** Group 4. Many antibody-forming cells are found in the cortico-medullary junction of regional lymph node. Six days after sensitization. Sandwich immunofluorescent method.  $\times 250$



**Photo. 6** Group 5. Massive proliferation of antibody-forming cells can be seen in the cortico-medullary junction and medullary cord of regional lymph node. Six days after sensitization. Sandwich immunofluorescent method.  $\times 250$