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Reactivation of juvenile hormone synthesis in adult drones of the honey bee, *Apis mellifera carnica*

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Abstract. Juvenile hormone synthesis was measured by the corpora allata radiochemical assay in vitro. No hormone was produced during pupal stages, but soon after adult eclosion the corpora allata were reactived. The rate of juvenile hormone III synthesis increased until day 10 after emergence. Possible functions of juvenile hormone in adult drones are discussed.
Key words. Juvenile hormone synthesis; honey bee drones; pupal stages; imaginal development; *Apis mellifera carnica.*

Studies on juvenile hormone in honey bees (*Apis mellifera*) have up to now focused on two functions: caste development[1–3] in late larval instars and during metamorphosis[4], and polyethism in adults[5,6]. Both functions concern female bees only; queen-worker differentiation in preimaginal development[3], and age-related worker tasks in the imago, called division of labor[7,8]. Drones have up to now received little attention in honey bee endocrinology[9]. To our surprise, the activity of the juvenile hormone-producing corpora allata during late larval stages in drones[10] resembled the situation in workers rather than that in the reproductively active females, the queens[11]. Data on juvenile hormone production of drones during the pupal phase and especially during adult development are still lacking and will be presented here for the first time.

Materials and methods

The rate of juvenile hormone production was measured in vitro[11] by the radiochemical assay for corpora allata activity[12], adapted for adult honey bees as previously described[13]. Only juvenile hormone III is produced by honey bees[14]. Three pairs of glands were coincubated for 6 h at 34.5 °C. The newly synthesized ^{14}C-labelled hormone was extracted from the medium with isooctane and separated by TLC[15]. The drone pupae and adults were removed immediately before dissection from strong, queenright summer colonies of *Apis mellifera carnica*. The staging of drone pupae, based on pigmentation of the eye and the mesothorax, was conducted every 4 h with the RAL color range card[16].

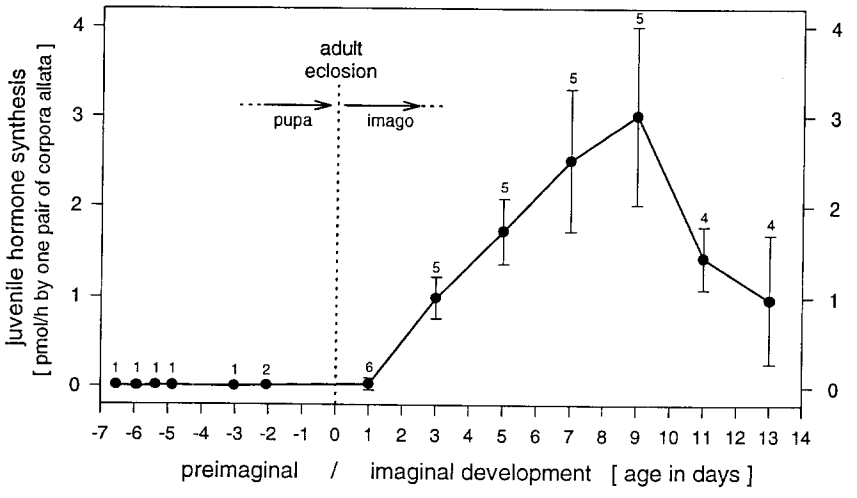

Figure. Developmental profile of corpora allata activity of *Apis mellifera carnica* drones as measured with the radiochemical in vitro assay. Mean rates of juvenile hormone III synthesis (±SEM) were calculated from 1–6 assays (see numbers on top of error bars), each run with 3 pairs of glands.

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Results

The drone corpora allata were completely inactive during all mid pupal and pharate adult stages until eclosion (fig.). Usually only one incubation per preimaginal stage was run since all the data were around the limits of detection of the radiochemical assay. Until the emergence of the adult drones, about one day after eclosion, no production of juvenile hormone was observed. However, only two days later a net synthesis of about one pmol/h of juvenile hormone III was measured per incubated pair of glands. This rate increased in a linear fashion for one week, reaching a peak of an average 3 pmol/h at 9 days after the imaginal moult. This peak was followed by a decline in the rate of juvenile hormone synthesis to about one pmol/h per pair of corpora allata at an imaginal age of two weeks (fig.).

Discussion

These data indicate that during the pupal phase of drone bee development there is no need for juvenile hormone functions. Immediately after the imaginal moult, however, in the adult drone the corpora allata become active again, as in the larval phase. In fact, 4 days after emergence an increased juvenile hormone III content had been determined in whole body extracts of drones[9]. The maximum rate of juvenile hormone III synthesis in imaginal drones is about 10-fold higher than that previously determined for the last larval instar during the early prepupal phase[10]. But what is the function of juvenile hormone in adult drones? In contrast to a large body of information on the gonadotropic role of juvenile hormone in female insects[17], little is known with regard to the possible contribution to male reproduction, especially control of sperm differentiation[18]. In adult worker bees, juvenile hormone synthesis also increases soon after the imaginal moult[13]. During the nurse phase there is a steady rise, but the highest titer of juvenile hormone III is found in the hemolymph of foragers[8]. Juvenile hormone application induces earlier flight activity than normal[19] and, therefore, juvenile hormone has been suggested to act as a flight stimulus[20].

The functions of juvenile hormone in adult drones of honey bees, as in other holometabolous insects[18], will include many of the processes occurring after emergence. Spermatogenesis begins during the prepupal phase[21], but sperm maturation[22], and also the production of large amounts of mucus by the male accessory glands[23], are observed only in the imaginal drone[24]. During the first week, adult drones stay in the colony and are fed by nurse-bee workers[25,26]. Around day 7–10 of imaginal life, short orientation flights are undertaken by the drones[27] which, after day 10, feed themselves on honey[26], especially after returning from energy-consuming mating flights[28] to drone congregation areas[29]. Thus, the imaginal phase of honey bee drones is also divided into a first, indoor period, and a second period with daily outdoors excursions, reflecting a premature phase[21,22] followed by full sexual maturity[24]. This lasts from day 12 until the end of a drone's life-span, or roughly 3–4 weeks after emergence[27,30]. The temporal sequence of drone in-colony and out-colony activities resembles the house-bee to field-bee sequence in a worker's life[31]. In which of these events juvenile hormone is involved, as a factor controlling the imaginal physiology and behavior of drone honey bees, is currently being investigated.

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The distribution of TM-316-associated surface antigen on polymorphonuclear leucocytes: an immunoelectron microscopic study

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Abstract. It has been established that MoAb TM-316 recognizes an epitope on leucocytes and specifically inhibits the chemotactic behavior of leucocytes. In the present paper, the distribution of this epitope on the cell surface and in intracellular organelles was studied by immunoelectron microscopy. Leucocytes separated from the blood of healthy men and from synovial fluid from patients suffering from rheumatoid arthritis were used. They were fixed with a mixture containing paraformaldehyde, glutaraldehyde and picric acid. As the second antibody, goat anti-mouse IgM conjugated to 10 nm gold colloids was employed.

In normal specimens, the epitope was found to some extent on the cytoplasmic membrane of neutrophilic leucocytes, but it was only sparsely distributed on eosinophilic and basophilic leucocytes. On activated neutrophilic leucocytes, obtained from the synovial fluid of rheumatoid arthritis patients, the immunolabeling was markedly increased. The number of sites where the epitope occurs on the surface of leucocytes is thus associated with the cell type, and also with the level of activation of the leucocytes.

In order to investigate the processing of the antigen, the intracellular localization of the epitope in the neutrophilic leucocytes was also studied. The epitope recognized by TM-316 was also detected in/on the characteristic granules and Golgi stacks.

Key words. Leucocyte; surface antigen; chemotaxis; immunocytochemistry.

As is well known, 'inflammation' includes hyperpermeability of blood vessels, migration of leucocytes and ingestion of cell débris by macrophages. Recently, various kinds of cytokines which stimulate cell proliferation have also been reported, and their properties were studied at the molecular level. However, research on cell migration – especially chemotaxis – has not progressed so rapidly. Cotter et al.[1] already reported that IgG1 anti polymorphonuclear-leucocyte monoclonal antibody inhibited chemotaxis towards zymosan-activated plasma. In 1988, Shimizu et al.[2] successfully prepared the monoclonal antibody (MoAb), termed TM-316, which inhibits the chemotaxis of neutrophile leucocytes, from the membrane protein of a human monocytoid leukemia cell line (THP-1). The antibody inhibited the chemotaxis induced by either complement, activated serum, N-formyl-L-methionyl-L-leucyl-L-phenylalanine, or the lymphocyte-derived chemotactic factor. However, it did not exert any influence on the number of Fc or complement receptors, lysosomal enzyme release, or adherence of leucocytes.

The distribution of the epitope which binds TM-316 on leucocytes has not been clarified. In the present paper, the authors describe the distribution of this antigen on various types of granulocytes as revealed by immunoelectron microscopy.

Material and methods

Polymorphonuclear leucocytes (PMN) were prepared from the peripheral blood of three healthy men and from synovial fluid of the knee joints of the three patients with rheumatiod arthritis. The procedures for obtaining specimens for immunoelectron microscopy were as follows. To collect leucocytes, heparinized blood was mixed with 3% dextran sulfate in physiological saline and kept at room temperature for 30 min after which the supernatant was layered on Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden) and centrifuged for 20 min to sediment the cells[2]. To collect basophilic leucocytes, the specific density gradient (Precoll, 1.068/1.076) method was applied as described previously[3], with a slight modification. The cell pellets were washed with physiological saline and then resuspended in a mixture of 4% paraformaldehyde, 0.2% picric acid and 0.5% glutaraldehyde in 8% sucrose solution buffered with 0.1 M phosphate (pH 7.4) for 4 h at 4 °C. After washing with 8% sucrose solution in 0.1 M phosphate buffer, the fixed leucocytes were centrifuged gently.

For the pre-embedding method of immunostaining, the sediment was resuspended in MoAb TM-316 diluted 60

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times with 0.1 M phosphate buffered saline containing 1% bovine serum albumin for 12 h at 4 °C. Then, after washing with 0.1 M phosphate buffer and centrifuging, the specimen was resuspended in goat anti-mouse IgM conjugated with 10 nm gold colloids (Zymed Lab., USA), diluted 100 times with 0.05 M Tris buffered saline containing 1% bovine serum albumin for 12 h at 4 °C. After washing three times with 0.1 M phosphate buffer and sedimenting gently, the cells were postfixed with 1% osmium tetroxide and embedded in Epon 812.

For post-embedding immunostaining, specimens fixed with the same mixture as for the pre-embedding method were dehydrated with a graded series of ethanols and embedded in LR-white at 53 °C overnight. Thin sections were prepared with the ultramicrotome and put on a nickel grid, and they were treated with anitbody (MoAb TM-316) prepared as mentioned above for several hours at 4 °C and then with gold conjugated goat anti-mouse IgM diluted in the same way for 4 h at room temperature. After washing thoroughly with 0.01 M phosphate buffer solution, they were stained with uranyl acetate and lead hydroxide, and observed with a JEM 2000EX electron microscope. In preparing the specimens, the authors made an effort to keep the thickness of the ultrathin sections constant (120 nm).

The best concentration of primary and secondary antibodies was determined by preliminary experiments. In control specimens, the primary antibody was omitted, or they were treated with gold-conjugated goat anti-mouse IgC as secondary antibody.

Observation

1. The immunoreaction of leucocytes in the preipheral blood. We first studied transverse sections of neutrophilic leucocytes with immunostaining before embedding.

Except for cells with damaged cytoplasmic membranes, a certain number of gold particles were recognized on the surface of neutrophilic leucocytes (figs 1 and 2). As shown in these figures, no background or unusual precipitation of gold particles could be found in sections. Antigenic sites labeled with gold particles against TM-316 were almost homogeneously distributed on the cell surface, especially on the surface coat (glycocalyx) lying a short distance (about 30 nm) above the cytoplasmic membrane (fig. 2). Occasionally, gold particles were arrayed in pairs and were attached in the same region, and the distribution density of particles was more intense around cytoplasmic processes (pseudopodia). The number of gold particles fluctuated in each cell, and roughly speaking, the particles on the surface coat were distributed at the rate of 8 per 1 μm of cytoplasmic membrane (fig. 2). The variation in the number of immunoreactive sites observed on each cell might be due to the difference in the preservation of antigenic sites during the process of fixation and staining. The thickness of specimens also influenced the number of labeling sites. No particles could be seen in the cytoplasm of the leucocytes.

Eosinophilic leucocytes were also recognized in the same sections. They were characterized by the specific profiles (crystals) of inclusions. After immunostaining, a few gold particles appeared on the surface of the eosinophilic leucocytes (fig. 3). The distance between antigenic sites was larger than that on neutrophilic leucocytes. The number of particles per 1 μm of the circumference of eosinophilic leucocytes was 2.3 (fig. 4).

As is well known, the ratio of basophilic leucocytes to all leucocytes is extremely low, and it was hard to recognize basophilic leucocytes in the same section. We therefore collected basophilic leucocytes with another density gradient method and examined the labeling on them. Basophilic leucocytes were iden-tified by their specific features, and the distribution of intracellular granules (fig. 5). On the surface of the cells, some gold particles were recognizable (fig. 6). The number of labeled sites was estimated as being almost similar to that of eosinophilic leucocytes.

Monocytes, erythrocytes and lymphocytes did not reveal any labelings on their surfaces. In the control specimen, the primary antibody was omitted, or anti-mouse IgG was used as secondary antibody. In both cases, no labeling was detected on the surface of the leucocytes.

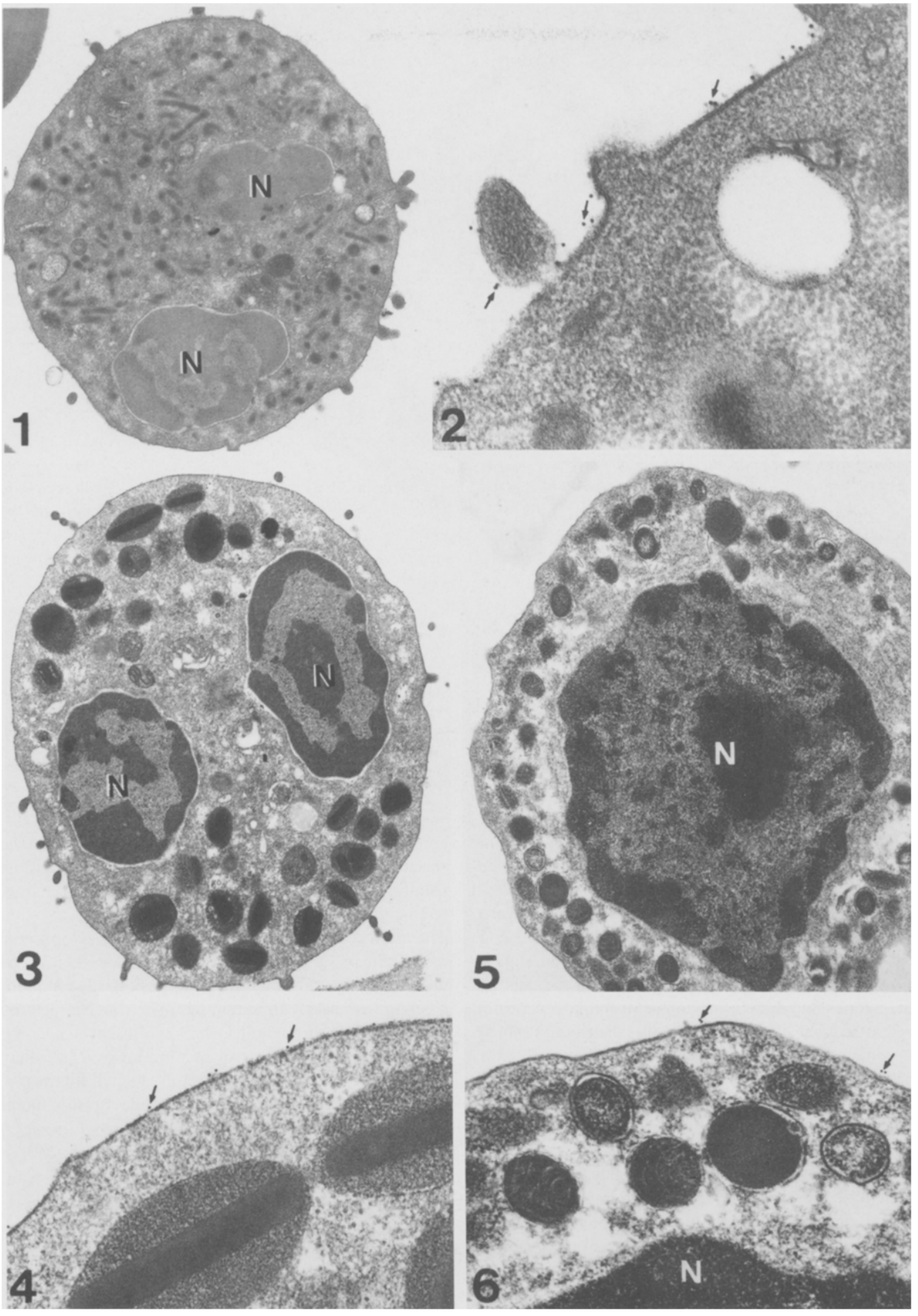
Figures 1–6. These electron micrographs show the gold particles (10 nm) revealed by MoAb TM-316 on granulocytes from normal individuals. Pre-embedding method.

Figure 1. General view of a neutrophilic leucocyte demonstrating a homogeneous distribution of gold particles over the whole surface the cell (N = nucleus). Magnification: ×13,000.

Figure 2. Gold particles on the surface coat of the leucocyte (arrows). No particles can be found in the cytoplasm. Sometimes, a pair of gold particles are observed. Uranyl acetate and lead hydroxide stain. Magnification: ×70,000.

Figures 3 and 4. Eosinophilic leucocytes showing characteristic inclusions with cristae. Some gold particles are scattered randomly on the cytoplasmic membrane (N = nucleus). In the higher magnification (fig. 4), one or two particles are sporadically distributed (arrows). Uranyl acetate and lead hydroxide stain. Magnification, fig. 3: ×11,000; fig. 4: ×45,000.

Figures 5 and 6. Basophilic leucocytes. Figure 5 indicates a distribution pattern of granules specific to basophilic leucocytes (N = nucleus). Several gold particles are found on the cytoplasmic membrane. Magnification: ×15,000. Figure 6 shows the unit membrane of cytoplasmic membrane and some gold particles (arrows) (N = nucleus). Uranyl acetate and lead hydroxide stain. Magnification: ×50,000.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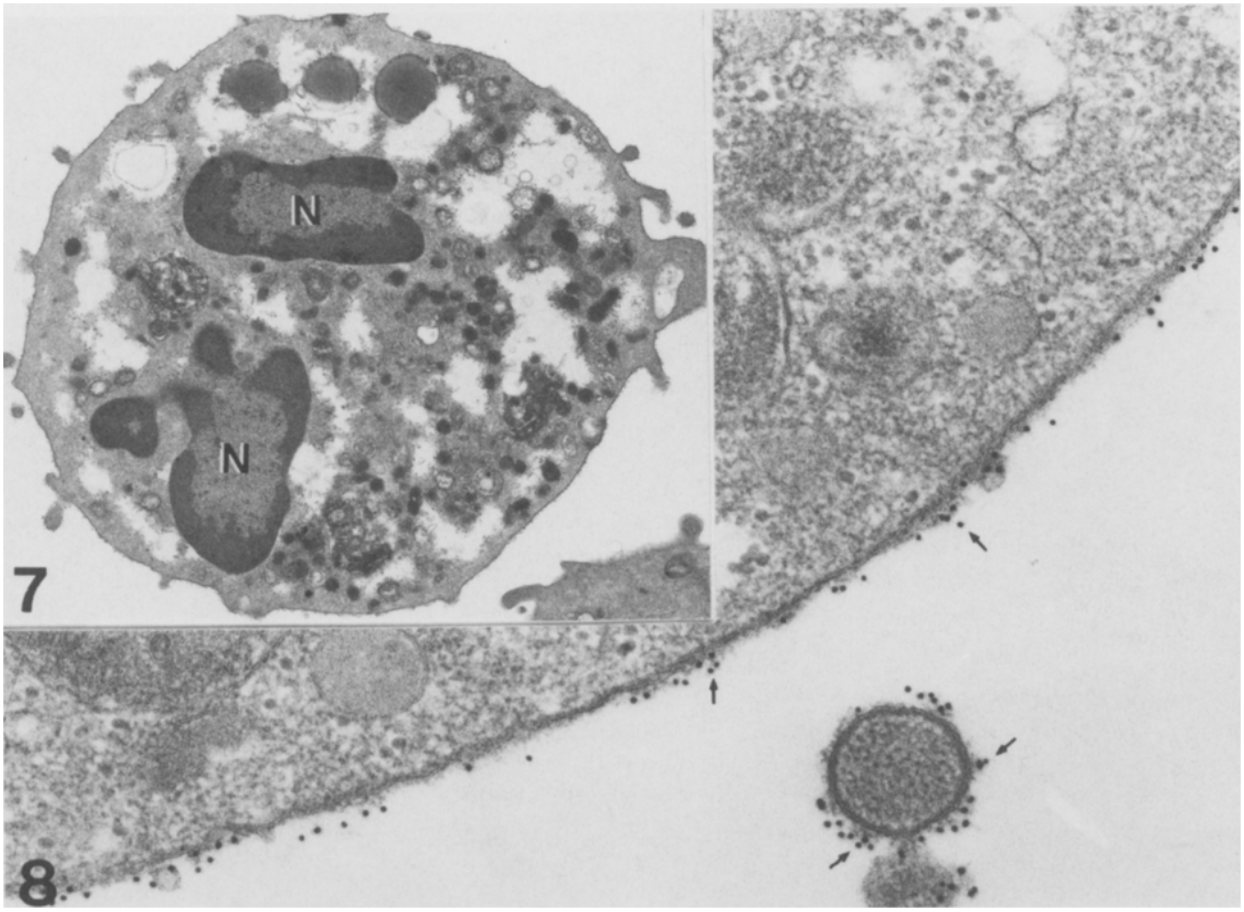


Figures 7 and 8. Electron micrographs showing cells from the synovial fluid of a patient suffering from rheumatoid arthritis. Pre-embedding method.
Figure 7. General view of a neutrophilic leucocyte (N = nucleus). On the cell surface, a lot of gold particles are distributed. Magnification: × 10,000.
Figure 8. In the more highly magnified picture (fig. 8), gold particles are arrayed regularly on the surface coat and in some places, especially on the cytoplasmic process, the particles are densely distributed (arrows). Uranyl acetate and lead hydroxide stain. Magnification: × 105,000.

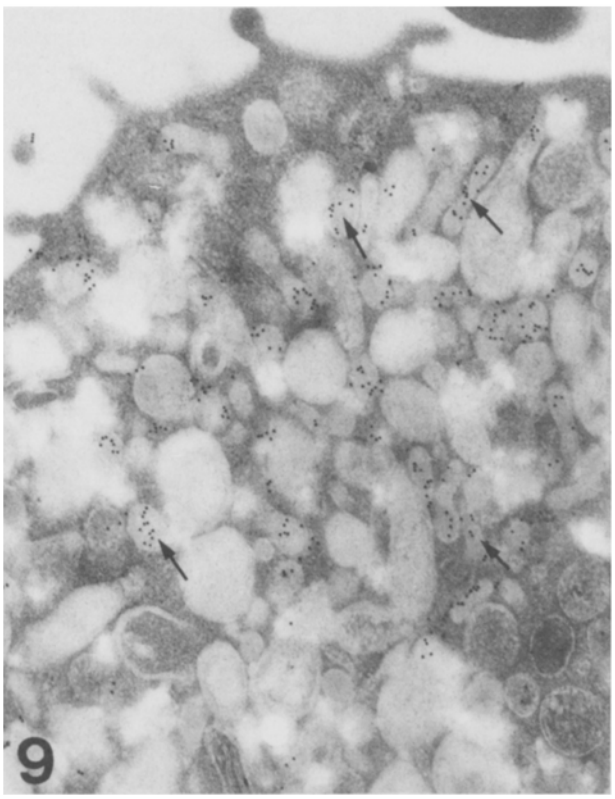
2. The immunoreaction of neutrophilic leucocytes in the synovial fluid of rheumatoid arthritis patients. Neutrophils in inflammed synovial fluid from rheumatoid arthritis patients were assumed to be in an activated state. That is, the cytosol of them looked heterogeneous and the characteristic granules were decreased (fig. 7). Occasionally, pseudopodia were evident on the cells. On the cytoplasmic membranes of neutrophilic leucocytes, a great number of gold particles was distributed (fig. 8). In some regions of these cells, the gold particles was thickly piled, but in the other regions of the same cells, they were relatively sparse. Roughly speaking, the amount of immunolabeling was several-fold more than on normal neutrophilic leucocytes.

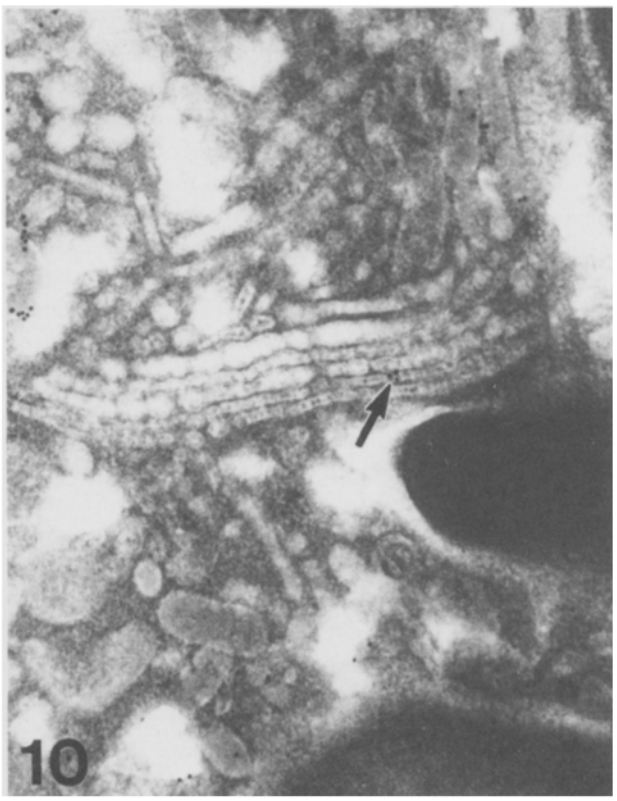
3. Intracellular distribution of immunolabeling in the neutrophilic leucocytes. The pre-embedding method described above was not suitable for this purpose, because the antigenic sites in the intracellular organelles were not accessible to the antibody. Thus, the post-embedding method described in 'Material and methods' was applied to the study of the intracellular distribution of epitopes in neutrophilic leucocytes. However, in the specimens embedded in LR-white resin the cytoplasmic membrane was not defined, and furthermore the characteristic granules looked pale (fig. 9).

The number of gold particles bound on the cytoplasmic membrane or surface coat was small. The reason will be discussed below. In the transverse section, the immunolabeling was clearly found along, or in, the characteristic granules throughout the cytoplasm (fig. 9). Occasionally, the gold particles were arrayed in a line. In the highly magnified micrograph, a small number of particles was detected on the cis side of the Golgi lamellae (fig. 10). The distribution of gold particles indicated the processing of antigen reacted with MoAb TM-316.

Discussion

The function of polymorphonuclear leucocytes is exerted at the site of inflammation and in the defense mechanism against endogeneous and exogeneous patho-





Figures 9 and 10. Intracellular distribution of gold particles reacted with MoAb TM-316 in neutrophilic leucocytes. Normal cells. Post-embedding method.
In figure 9, gold particles are mainly localized in the characteristic granules of neutrophilic leucocyte (arrows). Magnification: × 50,000. As shown in figure 10, gold particles are also found in some Golgi stacks, especially on the cis side of Golgi lamellae (arrow). Uranyl acetate and lead hydroxide stain. Magnification: × 60,000.

gens. The process of inflammation involves migration of leucocytes, including chemotaxis, and cell adhesion. On the other hand, it has been pointed out that in some patients the migration capacity of leucocytes is lost. Gahmberg et al.[4] and Bowen et al.[5] have reported on several cases of patients with defective PMN chemotaxis. According to them, leucocytes from patients had a decreased amount of glycoprotein on the cell surface. Shimizu et al.[2] reported that MoAb TM-316 inhibited chemotaxis induced by chemotaxic factors, but did not block PMN adhesion to nylon fibers. They have thus speculated that MoAb TM-316 is capable of binding to the epitope near the active site of migration, probably by blocking a chemotactic receptor and/or by exerting some influence on the cell metabolism.

As is well known, neutrophilic leucocytes are the first cells to be mobilized into the lesion under conditions of inflammation[6]. If chemotactic migration of PMN is closely associated with the epitope reacted with MoAb TM-316[2], it is to be expected that the number of sites immunolabeled with gold particles on neutrophilic leucocytes would be greater than the number on eosinophilic or basophilic leucocytes, and furthermore, in the synovial fluid of arthritis patients, where neutrophils are activated, the number of epitope sites on their surfaces would be markedly increased. The results obtained by the present study have confirmed this speculation.

The technique employed requires some discussion. Immunohistochemical methods have advanced recently and have been successful in demonstrating the localization of macromolecules at the light and electron microscope levels. The protein A gold method, tagged with various markers, was introduced by Bächi et al.[7] and Roth et al.[8,9], and has been found to be reliable and to have a wide range of applications. However, as pointed out by Bendayan[10], there are some pitfalls in this method. Therefore, the present authors were careful to select a suitable concentration of antibody for preventing background staining, appropriate components of fixatives for the preservation of antigenicity, and constancy in the thickness of the sections.

However, it was still found that the number of epitope sites observed on the surface of leucocytes varied greatly between cells. The differences may be related to the aging of the leucocyte, the penetration of antibody and/or the fixation procedures used. For a quantitative study of labeling on leucocytes, the observations would need to be carried out over a larger area. The scanning electron microscope could be used[11-13].

When the immunoreactivity of leucocytes prepared by the pre-embedding and post-embedding procedures was compared, the former gave better results for the surface epitope, whereas the latter was more suitable for detecting the intracellular epitope. The difference was due to the permeability to antibody, and to the fixatives and the resin used for embedding. In the post-embedding method, the immunolabeling was restricted to the surface of the sections, and only those epitopes revealed in cut surfaces of the cells could react with the antibody[10].

The cytoplasmic organelles that reacted with MoAb TM-316 were identified on the basis of size, electron density and distribution. The gold particles were found to be distributed in the regions corresponding to the sites of the characteristic granules and, in some specimens, in the stacks of Golgi lamellae of neutrophilic leucocytes. It is interesting to note here that, according to Dunphy and Rothman[14], Golgi lamellae play a significant role in the adding of saccharide chains to the core protein, which is produced by the endoplasmic reticulum. The study of the distribution of the epitope reacting with MoAb TM-316 on leucocytes is a further step in understanding the mechanism of leucocytic chemotaxis under normal and pathological conditions.

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