Ultrastructural changes in follicles of small-intestinal aggregated lymphoid nodules in early and advanced phases of experimentally induced mucosal disease in calves

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Objective—To investigate ultrastructural changes in follicles of small-intestinal aggregated lymphoid nodules (Peyer’s patches) of calves with early and advanced phases of experimentally induced mucosal disease (MD).

Animals—Twenty 2.5- to 7-month-old Holstein-Friesian calves (11 females, 9 males).

Procedure—MD was induced in 13 of 18 calves that were persistently viremic with bovine viral diarrhea virus (BVDV). Eight of the 13 calves were euthanized before the onset of clinical signs of MD, and 5 were euthanatized after becoming moribund with MD. Five persistently viremic calves and 2 calves without BVDV served as controls. Specimens of small-intestinal aggregated lymphoid nodules were prepared for transmission electron microscopy.

Results—The ultrastructure of follicles of small-intestinal aggregated lymphoid nodules from healthy calves was consistent with that in sheep. In the early phase of MD, changes were characterized by numerous apoptotic lymphocytes and macrophages with apoptotic bodies. In more advanced lesions, affected lymphoid follicles consisted of macrophages and variable numbers of follicular dendritic cells (FDC), whereas others did not contain FDC. In moribund calves, small follicles consisting predominantly of FDC and follicles with central cavities surrounded by macrophages, and few neutrophils were observed.

Conclusions and Clinical Relevance—The ultrastructural changes in lymphoid follicles of small-intestinal aggregated lymphoid nodules indicate apoptosis of lymphocytes as an initial event. The development of small follicles consisting predominantly of FDC or the complete loss of follicular architecture in advanced phases of MD is determined by the intensity of apoptosis of lymphocytes, the capacity of the macrophages for uptake, and the reorganization of a stromal network. (Am J Vet Res 2000;61:174–182)

Mucosal disease (MD) represents a distinct course and consequence of the infection with bovine viral diarrhea virus (BVDV), a pestivirus from the genus flaviviridae. Under experimental conditions, MD may be induced when cattle that are persistently viremic with noncytopathic BVDV are inoculated with an antigenically homologous cytopathic BVDV. After the onset of diarrhea, cattle succumb rapidly. At necropsy, fibrinous to ulcerative enteritis, which is most severe at sites overlying small-intestinal aggregated lymphoid nodules (ie, Peyer’s patches) and mucosa-associated lymphoid nodules in the large intestine, is a characteristic finding in cattle moribund with MD.

After experimental intranasal infection of persistently viremic cattle, cytopathic BVDV replicates primarily in the tonsils. From there it spreads to lymphatic tissues including small-intestinal aggregated lymphoid nodules. Cytopathic BVDV antigen is first found in single or small groups of follicles, later extending to all follicles. In early infection, cytopathic BVDV is found in lymphocytes as well as in cells with dendritic morphologic characteristics within aggregated lymphoid follicles. Alterations in lymphoid follicles containing cytopathic BVDV are characterized by patchy to diffuse areas with low proliferation and high apoptosis. In moribund cattle in the advanced phase of MD, the severely depleted lymphoid follicles mostly consist of a network of cells with dendritic morphologic characteristics that are positive for cytopathic BVDV. These lymphoid follicles are markedly reduced in size and contain few proliferating and apoptotic cells. Lymphoid follicles of small-intestinal aggregated lymphoid nodules are localized in the submucosa. As in secondary follicles of other lymphoid tissues, they can be subdivided into a dark outer zone with high numbers of proliferating lymphocytes and a light inner zone with less proliferation. The lymphoid follicles consist predominantly of B lymphocytes, less CD4+, and few CD8+ T lymphocytes. Cells resembling tingible body macrophages are interspersed. Between the lymphocytes and macrophages, a stromal network of cells with dendritic morphologic characteristics is observed. These cells have been identified as follicular dendritic cells (FDC) in other lymphoid tissues. Follicular dendritic cells are defined by their morphologic characteristics, their exclusive presence within lymphoid follicles, and their ability to retain immune complexes on their surface for long periods. Follicular dendritic cells and B lymphocytes depend on each other for survival.

The purposes of the study presented here were to...
describe the ultrastructure of lymphoid follicles of small-intestinal aggregated lymphoid nodules in clinically healthy calves without BVDV infection and with persistent viremia and to demonstrate the successive ultrastructural changes in calves after experimental induction of MD by inoculation with cytopathic BVDV.

Materials and Methods

Calves—Twenty 2.5- to 7-month-old Holstein-Friesian calves (11 females and 9 males) from 3 herds were used (Table 1). Eighteen calves were persistently viremic with herd-specific noncytopathic BVDV, and 2 were not.

Experimental protocol—Antigenically homologous cytopathic BVDV were selected on the basis of epitopic mapping, using E2-glycoprotein-specific monoclonal antibodies. Eight of the persistently viremic calves were inoculated intranasally with matching cytopathic BVDV and euthanatized in the early phase of MD at days 5, 7, 9, and 13 after inoculation and before the onset of diarrhea. Five calves were inoculated and euthanatized in the advanced phase of MD when they were moribund.

Five of the persistently viremic calves were not inoculated with cytopathic BVDV. They were healthy at the time of euthanasia and necropsy and served together with the 2 calves that were free of BVDV, as determined by virus isolation from blood samples, as control calves.

Collection and preparation of tissues—Calves were anesthetized by IV administration of ketamine hydrochloride (4 to 8 mg/kg of body weight) and xylazine hydrochloride (1 mg/kg). After laparotomy in the left flank, intestinal loops from midjejunum with small-intestinal aggregated lymphoid nodules and ileum adjacent to the ileocecal entrance were ligated and filled with a 2.5% solution of glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). Then the calves were euthanatized by IV administration of an aqueous solution that contained 0.04 g tetracaine hydrochloride b (1 mg/kg). After laparotomy in the left flank, intestinal loops from midjejunum with small-intestinal aggregated lymphoid nodules and ileum adjacent to the ileocecal entrance were ligated and filled with a 2.5% solution of glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). Then the calves were euthanatized by IV administration of an aqueous solution that contained 0.04 g embutramide, 0.01 g mebenzonium iodide, and 0.001 g tetracaine hydrochloride/kg, and the ligated small intestine was removed. From these loops, tissue specimens of 1 cm² were excised, cut into small squares of 1 mm², and transferred to fresh fixative for 24 hours at 4 C. They were further fixed in 1% solution of osmium tetroxide, dehydrated in ethanol, and embedded in epon. Semithin sections (1 μm thick) were stained with toluidine blue and examined by light microscopy. From selected areas, ultrathin sections (90 nm thick) were cut, contrasted with uranyl acetate and lead citrate, and examined with a transmission electron microscope.

Results

Control calves—On light microscopy, lymphoid follicles of jejunal aggregated lymphoid nodules from control calves (No. 1 to 7) were round, whereas those of lymphoid follicles of ileal aggregated lymphoid nodules were larger and ovoid shaped (Fig 1). The overlying corona and dome limited the apical part of the lymphoid follicle that could be subdivided into a dark-staining outer, and a light-staining inner zone. The basal part was surrounded by a thin capsule of connective tissue.

On transmission electron microscopy, the outer zone of the lymphoid follicles consisted of numerous closely associated lymphocytes with slightly indented, heterochromatic nuclei (Fig 2) and several macrophages that sometimes contained phagocytosed material. Mitotic figures were common. The stromal network was formed by FDC with numerous long and slender cell processes branching from cell bodies that were rarely observed. The euchromatic nuclei of FDC were round to ovoid and contained a central nucleolus. Some mitochondria, polyribosomes, and a moderately developed rough endoplasmic reticulum were observed in the narrow perinucleus space. The cell processes contained intermediate filaments and polyribosomes (Fig 3). Their terminal endings were connected by desmosomes.

In the inner zone, lymphocytes with round, euchromatic nuclei were loosely arranged.

Table 1—Protocol for experimentally induced mucosal disease in calves

<table>
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*Indiana and MD-1 are distinct strains of BVDV. 1Indiana CK40 is a biological clone of BVDV strain Indiana.

Group 1 = 2 healthy calves without viremia. Group 2 = 5 healthy, persistently BVDV viremic calves. Group 3 = 8 Persistently BVDV viremic calves in the early phase of mucosal disease (MD). Group 4 = 5 Persistently BVDV viremic calves in the advanced phase of MD. BVDV = Bovine viral diarrhea virus. NI = Not inoculated. NA = Not applicable.
Macrophages and mitotic figures were detected less commonly than in the outer zone. In contrast to FDC of the outer zone, those of the inner zone had numerous mitochondria and well-developed rough endoplasmic reticulum and Golgi system. The cell processes that were multifocally embedded in homogeneous, electron dense material terminated in aggregations of small convoluted dendrites connected by desmosomes.

The lymphoid follicles were surrounded by a capsule of connective tissue formed by ≤ 6 layers of fibroblasts and collagen fibrils measuring 50 to 80 nm in diameter. Small aggregations of thin collagen fibrils with a diameter of 20 to 30 nm were observed between the cells within the lymphoid follicles. There were no morphologic differences between tissue specimens from calves without BVDV (No. 1 and 2) and persistently viremic calves (No. 3 to 7).

Calves with mucosal disease—In calves in the early phase of MD (No. 8 to 15), ultrastructural changes were observed first in single follicles and then extended to groups of follicles, which was comparable with the tissue distribution of cytopathic BVDV antigen. In calves in the advanced phase of MD (No. 16 to 20), all lymphoid follicles were severely altered. The extent and degree of alterations differed between lymphoid follicles of the jejunum and ileum in individual calves, with more severe lesions found in the ileum.

On the basis of the degree of lymphocyte depletion and the reduction in size of the lymphoid follicles in calves with MD, compared with control calves, ultrastructural lesions were classified as types A to E, with type-A lesions representing the least severe findings and type-E lesions representing the most severe findings. For some specimens, multiple classifications were applied because of varying types of alterations in the lymphoid follicles of aggregated lymphoid nodules (Table 2).

Type-A lesions—Most calves in the early phase of MD had type-A lesions (Table 2). On light microscopy, lymphoid follicles from these calves were slightly depleted of lymphocytes, but the follicles were not reduced in size, compared with control calves. Commonly, the depleted areas had a patchy
distribution, affecting predominantly the outer zone of the apical part of the lymphoid follicle (Fig 4). Because of the depletion of lymphocytes, outer and inner zones were difficult to distinguish. The number of mitotic figures was low.

On transmission electron microscopy, depleted areas associated with type-A lesions contained several lymphocytes with deep indentations of the heterochromatic nuclei. A high number of electron-dense, shrunken lymphocytes with clumped chromatin that sometimes formed crescents along the inner nuclear membrane, but without changes of the cellular organelles, were observed (Fig 5). They were interpreted as apoptotic cells. Small apoptotic bodies were commonly found between lymphocytes. Numerous macrophages with extending cytoplasm were observed containing high numbers of apoptotic cells in varying degrees of digestion. Follicular dendritic cells were more commonly observed in calves with type-A lesions than in control calves. Morphologic characteristics of FDC of calves with type-A lesions were comparable to the appearance of FDC in the inner zone of lymphoid follicles of control calves. In 2 calves in the early phase of MD (No. 10 and 12), a few FDC with electron-dense, deeply indented nucleus, electron-dense cytoplasmic matrix, and variable amounts of endoplasmic reticulum and Golgi system were observed in the depleted areas of some lymphoid follicles.

Type-B lesions—Many calves in the early phase of MD had type-B lesions (Table 2). On light microscopy, lymphoid follicles from these calves were depleted, but only slightly reduced in size (Fig 6), compared with control calves. The differentiation between outer and inner zones was not possible. Wide intercellular spaces separated the remaining lymphocytes, apoptotic cells, and prominent macrophages. In the subcapsular area, small clusters of intact lymphocytes were observed. Some mitotic figures were observed.

On transmission electron microscopy, type-B lesions were characterized by numerous apoptotic lymphocytes in varying stages of death, apoptotic

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* A = Mild, often patchy depletion of lymphoid follicles with numerous apoptotic cells. Macrophages contain numerous apoptotic bodies and cells. B = Severely depleted lymphoid follicles with numerous apoptotic cells. Macrophages contain apoptotic bodies and residual bodies. C = Severely depleted lymphoid follicles containing predominantly macrophages, few lymphocytes, and variable numbers of follicular dendritic cells. D = Small, severely depleted lymphoid follicles consisting predominantly of follicular dendritic cells. E = Small lymphoid follicles with central cavities. The remnant tissue consists predominantly of macrophages and a few neutrophils. tAlterations were found in some (+), numerous (++), or all (+++) small-intestinal aggregated lymphoid follicles.

Table 2—Severity and extent of lesions of small-intestinal aggregated lymphoid nodules in calves with experimentally induced early (group 3) and advanced (group 4) mucosal disease

![Figure 4](image-url) Photo-micrograph of a section of ileal aggregated lymphoid nodules from a persistently viremic calf (No. 12) in the early phase of mucosal disease (MD) with characteristic type-A lesions. Notice the 2 patchy areas of depletion (D) in the apical part of the follicle. An outer (O) and inner (I) zone can be distinguished in the basal part of the follicle. Semithin section, toluidine blue stain. Bar = 100 μm.

![Figure 5](image-url) Electron micrograph of an area of lymphocyte depletion of an ileal lymphoid follicle from a persistently viremic calf (No. 10) in the early phase of MD with characteristic type-A lesions. Notice that many lymphocytes are in varying phases of apoptosis with shrinkage and accumulation of chromatin in the periphery of the nucleus, detachment of the inner nuclear membrane (arrowheads), and condensation of karyoplasmin and cytoplasm (*). Numerous apoptotic cells and apoptotic bodies have been phagocyted by macrophages (M). A morphologically unaltered FDC (F) is found between the apoptotic cells. Bar = 4 μm.
bodies, and macrophages with large, irregularly shaped, euchromatic nuclei (Fig 7). The latter contained, in addition to apoptotic bodies, intracytoplasmic vacuoles with electron dense, granular, or unstructured material. Only single FDC were observed in the depleted areas. In contrast, FDC were common in the subcapsular areas where small groups of lymphocytes were observed. Cells with short and shrunken cell processes were observed, which were identified as altered FDC on the basis of their elongated shape and high contents of intermediate filaments.

**Type-C lesions**—Some calves in the early phase of MD and 1 calf in the advanced phase of MD had type-C lesions (Table 2). On light microscopy, the severely depleted lymphoid follicles were moderately reduced in size (Fig 8), compared with control calves. Within the lymphoid follicles, round macrophages predominated along with a few lymphocytes and single neutrophils. Mitotic figures were not observed.

On transmission electron microscopy, type-C lesions had macrophages with well-developed cellular organelles but few lysosomes in the electron lucent cytoplasmic matrix (Fig 9). Apoptotic bodies were rarely observed within macrophages and extracellularly. There was an obvious lack of lymphocytes.

In calves with type-C lesions, FDC were observed in varying numbers. Follicular dendritic cells could not be distinguished in follicles of the jejunal and ileal aggregated lymphoid nodules of 1 calf (No. 13) and of the jejunum only of another calf (No. 15). In contrast, some or even numerous FDC were observed within follicles of the ileal aggregated nodules of 1 calf (No. 15) in the early phase of MD and within follicles of the jejunal and ileal aggregated lymphoid nodules of 1 calf (No. 16) in the advanced phase of MD. These FDC had well-developed organelles. Sometimes the nuclei of FDC had small indentations. The cell processes contained numerous intermediate filaments. There were no terminal convoluted dendrites, and the number of desmosomes was reduced, compared with control calves. Some FDC were observed adjacent to blood vessels.
Type-D lesions—Most calves in the advanced phase of MD had type-D lesions (Table 2). On light microscopy, severely depleted lymphoid follicles from these calves were reduced in size, compared with control calves. They consisted of closely associated round to elongated cells with large euchromatic nuclei and clear cytoplasm (Fig 10). Mitotic figures were not observed.

On transmission electron microscopy, type-D lesions contained lymphoid follicles that comprised predominantly FDC, low numbers of macrophages, and single scattered lymphocytes (Fig 11). Follicular dendritic cells contained numerous mitochondria and had well-developed rough endoplasmic reticulum and Golgi systems in the perinuclear space and in some instances even in their cell processes. Several FDC had slightly indented nuclei. The number of desmosomes between the convoluted terminal endings of cell processes was low. Macrophages
had the same morphologic characteristics as described for type-C lesions.

Type-E lesions—Two calves in the advanced phase of MD (No. 16 and 19) and 1 calf in the early phase of MD (No. 15) had type-E lesions (Table 2). On light microscopy, small lymphoid follicles were characterized by central cavities that were surrounded by tissue severely depleted of lymphocytes (Fig 12). The central cavity was sometimes lined by epithelium.

On transmission electron microscopy, type-E lesions had remnant subcapsular tissue consisting mainly of macrophages, as described for type-C lesions, and some neutrophils (Fig 13). Follicular dendritic cells were not observed. Central cavities contained a few macrophages and apoptotic bodies. The macrophages had an electron lucent cytoplasmic matrix with numerous vesicles and vacuoles. In some lymphoid follicles, the cavities were lined by 1 layer of flattened epithelial cells with short microvilli. Epithelial cells sometimes had condensed cytoplasm and karyoplasm.

Discussion

Bovine viral diarrhea virus induces severe lesions in lymphoid follicles, including small-intestinal aggregated lymphoid nodules. The ultrastructural alterations observed in this investigation support further interpretations of histologic and immunohistologic findings.

Primarily lymphoid follicles of small-intestinal aggregated lymphoid nodules of healthy calves without BVDV were examined, because their ultrastructure has not been described in the literature to our knowledge. The morphologic characteristics of lymphocytes and macrophages in the small-intestinal aggregated lymphoid follicles of the calves examined were consistent with those reported from lymphatic tissues of humans, mice, and sheep. The nuclei of FDC in the lymphoid follicles of small-intestinal aggregated lymphoid nodules of calves were round, ovoid or elongated in shape, euchromatic, and with a smooth nuclear membrane. This is comparable with FDC described in lymphoid follicles of small-intestinal aggregated lymphoid nodules of sheep but differs from FDC in lymph nodes and tonsils of humans and laboratory animals, where the nuclei are described as lobulated, indented, multinucleated, and heterochromatic. It is unclear whether this difference in nuclear morphologic characteristics is organ specific or species specific for ruminants. Differences in the contents of organelles and morphologic characteristics of cell processes have been used to classify FDC of variable degrees of maturation within compartments of the lymphoid follicles of human tonsils. Although subtyping into the 7 proposed categories of FDC was not quite feasible, immature FDC of the outer zone and mature FDC of the inner zone of lymphoid follicles of small-intestinal aggregated lymphoid nodules of calves could be distinguished. The observation of 2 phenotypes of cell processes of FDC, namely filiform and beaded, as described for mice, could not be observed in cattle.

No morphologic differences were found between the lymphoid follicles of the calves without BVDV and the 5 healthy calves persistently viremic with noncytopathic BVDV. This is consistent with other histologic and immunohistologic findings in lymphoid tissues of persistently viremic cattle. Thus, the calves without BVDV and the 5 healthy persistently viremic calves were used as controls for our ultrastructural study.

The objective of this investigation was to demonstrate the alterations in the lymphoid follicles of small-intestinal aggregated lymphoid nodules during the early and advanced phase of MD. This was achieved by including persistently viremic calves, euthanatized at various predetermined times after inoculation with cytopathic BVDV.

On the basis of findings in our study, we hypothesize that the initial change includes extensive cell death with the morphologic features of apoptosis. The first focal, then diffuse, distribution of this lesion confirms previous investigations and is consistent with the distribution of cytopathic BVDV. Predominantly lymphocytes were affected by apoptosis, because their number was severely reduced, and a high number of FDC and macrophages were found between the apoptotic cells. Thus, it can be concluded that cytopathic BVDV, which can be detected in lymphocytes as well as in association with FDC, has varying effects on these cells. Follicular dendritic cells are most likely not destroyed in this phase of infection, because they retain particles of the cytopathic BVDV on their plasma membrane only, without internalization. This would correspond with experimental findings in laboratory animals that indicated that, under physiologic conditions, FDC trap and retain native antigen on their plasma membrane and contribute to the immune response by presenting the trapped antigens to germinal center B lymphocytes. On the other hand, differentiated FDC may not be destroyed, because they do not proliferate.

Macrophages with phagocytosed apoptotic cells were observed in lymphoid follicles with numerous apoptotic cells as well as in depleted lymphoid folli-
cles. Rapid uptake of apoptotic cells by macrophages and adjacent cells has been described.\(^6\) This is mediated by structurally distinct molecules specifically expressed on the surface of apoptotic cells that are recognized by receptors on phagocytes.\(^6\) Macrophages that contained predominantly residual bodies were observed in more progressed lesions, because digestion of apoptotic cells proceeds quickly after phagocytosis.\(^2\) Despite efficient removal by macrophages, numerous apoptotic bodies were observed extracellularly. This indicates an extremely high rate of cell death, which exceeds the capacity of macrophages for uptake. Apoptotic bodies that were not phagocytosed may undergo secondary necrosis.\(^9\) This may attract neutrophils that were found in low numbers in some of the severely depleted lymphoid follicles and in the remnants of lymphoid tissue around the cystic cavities. The resulting secondary necrosis may explain the severe tissue destruction observed in the center of these lymphoid follicles.

There were also changes in the morphologic characteristics and distribution of FDC. Numerous FDC containing prominent cell organelles were initially observed in the outer zone of lymphoid follicles from calves with MD, indicating cellular activation, compared with control calves. This may be induced by binding of viral particles on the cell surface of the FDC, because binding of antigen results in the expression of adhesion molecules and Fc receptors, a process that requires extensive cellular organelles.\(^\) Follicular dendritic cells were not observed in the centers of severely depleted lymphoid follicles. This loss of FDC was interpreted as consequence of the depletion of B lymphocytes, because FDC and B lymphocytes depend on each other for survival.\(^1\) Despite the depletion, small groups of lymphocytes mixed with FDC were found in the subcapsular region. Some of these FDC had short cell processes and condensed cytoplasm and karyoplasm that were interpreted as degenerative changes. Comparable alterations have been reported and interpreted as unspecific signs of degeneration in germinal centres of spleen and lymphocytes.\(^2\) This indicates an extremely high rate of cell death, which exceeds the capacity of macrophages for uptake. Apoptotic bodies that were not phagocytosed may undergo secondary necrosis.\(^9\) This may attract neutrophils that were found in low numbers in some of the severely depleted lymphoid follicles and in the remnants of lymphoid tissue around the cystic cavities. The resulting secondary necrosis may explain the severe tissue destruction observed in the center of these lymphoid follicles.

High numbers of activated FDC were observed in some severely depleted lymphoid follicles from calves in the advanced phase of MD. This was interpreted as repopulation by FDC that may be induced and modulated by the cytokine GM-CSF secreted by macrophages\(^5\) or the ligation of the surface molecule CD40 by T lymphocytes.\(^5\) This repopulation may have originated from the small subcapsular foci of cells containing FDC. The close association of some FDC with blood vessels may indicate that these are used as guiding devices. A successful repopulation of FDC will result in the formation of a dense stromal network as observed in some lymphoid follicles of moribund calves. Lymphoid follicles that contain predominantly stromal cells after depletion of B-lymphocytes by dex-athemase have been reported to be repopulated by lymphocytes.\(^1\)

In some lymphoid follicles of 2 calves that were euthanatized in the advanced phase of MD (No. 16 and 19) and 1 calf in the early phase of MD (No. 15), no repopulation by FDC was evident. Instead, a complete destruction of the follicular architecture with formation of a central cavity was observed. Reduced growth activity of FDC may be the result of the inhibiting effect of cytokines like interleukin 1 and tumor necrosis factor that can also be secreted by macrophages\(^6\) or a cytotoxic effect of BoCD4+ T lymphocytes.\(^6\) In addition, extensive secondary necrosis may contribute to the complete follicular breakdown.\(^2\) Thus, the final outcome of small condensed lymphoid follicles consisting mainly of FDC versus lymphoid follicles with central cavities will be determined by the intensity of the apoptosis of lymphocytes and the capacity of the macrophages to phagocytose and remove apoptotic cells as well as to mediate the repopulation by FDC.

\(^{1}\)Ketamin, WDT, Garbsen, Germany.
\(^{2}\)Rompun, Bayer, Leverkusen, Germany.
\(^{3}\)T61, Hoechst Veterinar GmbH, Unterschleißheim b. München, Germany.
\(^{4}\)EM 10C, Zeiss, Oberkochen, Germany.

References


