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# Selective double staining of interstitial cells of Cajal and macrophage-like cells in small intestine by an improved supravital methylene blue technique combined with FITC-dextran uptake*

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**Summary.** By a brief exposure of small intestine (mouse, rat, guinea pig) to lysolecithin prior to vital methylene blue staining we were able to demonstrate in a selective way the complete network of interstitial cells of Cajal, located in the space between the longitudinal and circular muscle layers. A combination with fluorescence labeling (FITC-dextran uptake) of macrophage-like cells, allowed us to demonstrate 1) the complete, regular distribution of both cell populations along the entire small intestine, and 2) the constant, intimate associations between interstitial cells of Cajal and macrophage-like cells.

In relation to current hypotheses concerning a role of interstitial cells of Cajal in motility regulation, our results call attention to the possible involvement of another cell type, the macrophage-like cell.

**Key words:** Interstitial cells of Cajal – Macrophage-like cells – Double staining method – Vital methylene blue – Small intestine

## Introduction

A regulatory role of interstitial cells of Cajal (ICC) in gastrointestinal motility is currently under investigation (Daniel 1977; Rumessen and Thuneberg 1982; Rumessen et al. 1982; Thuneberg 1982, 1988; Durdle et al. 1983; Faussone-Pellegrini and Cortesini 1983; Daniel and Posey-Daniel 1984; Hara et al. 1986; Suzuki et al. 1986; Smith et al. 1987). A major problem is an unambiguous identification of ICC. One of the few available methods is the supravital methylene blue staining (Cajal 1893; Taxi 1965; Thuneberg 1982). This method is basically nonspecific, but nevertheless certain modifications can produce highly selective staining patterns. The staining usually is incomplete, in so far as the uptake of dye is restricted to smaller or larger patches of the interstitial cell network.

Using the small intestine from mice, rats and guinea pigs, we have further developed the methylene blue method to facilitate a nearly-selective staining of the *entire* network of ICC associated with Auerbach's plexus (ICC-AP). This modification involves a preincubation with lysolecithin causing destruction of a mesothelial diffusion barrier.

We have previously shown (Mikkelsen et al. 1985) that the interstices around Auerbach's plexus contain a population of macrophage-like cells (MLC-AP) in strikingly constant numbers and distribution throughout the small intestine. Our ultrastructural studies show areas of close association between ICC-AP and MLC-AP.

The present study is inspired by the work of Taxi (1965). Taxi clearly distinguished between ICC and histiocytes (MLC) of guinea pig and mouse small intestine by using a combination of methylene blue staining of ICC and trypan red uptake by histiocytes (MLC).

To show the potentials of the improvement of the methods we demonstrate by a double-labeling technique – methylene blue staining of ICC-AP, combined with uptake of FITC-dextran by MLC-AP – the regular and constant association of the two cell types.

## Materials and methods

Albino mice, (suckling and adult), Wistar rats and guinea pigs were used. The animals were killed by cervical dislocation, the small intestine was carefully removed and immersed in $7 \cdot 10^{-4}$ M lysolecithin in calcium-free, phosphate buffered saline (PBS). In some experiments the intestine was exposed to lysolecithin for 2–10 min, followed immediately by methylene blue staining (see below). In other experiments the lysolecithin treatment was restricted to 10–15 s followed by immersion for 1 h in medium 199 (Gibco) supplemented with $4 \cdot 10^{-3}$ M $CaCl_2$, $2 \cdot 10^{-3}$ M $MgCl_2$, prior to methylene blue staining.

The tissue was transferred to aerated medium 199, supplemented with $4 \cdot 10^{-3}$ M $CaCl_2$ and $2 \cdot 10^{-3}$ M $MgCl_2$, and containing methylene blue B (Merck) $1 \cdot 10^{-5}$ M. Staining was monitored by stereo microscopy, the optimal exposure time being about 30 min. The whole procedure was carried out at room temperature (20–22° C). Tissue was fixed in 2% formaldehyde, 15% picric acid in PBS (Stefanini et al. 1967). For microscopy whole-mounts of the intestinal wall or the isolated muscularis were prepared. Routine sections for light and electron microscopy (Mikkelsen et al. 1985) confirmed that after lysolecithin and methylene blue exposure the mesothelium was absent or severely damaged, with little or no structural change in the superficial muscle cells.

For double-labeling experiments animals received

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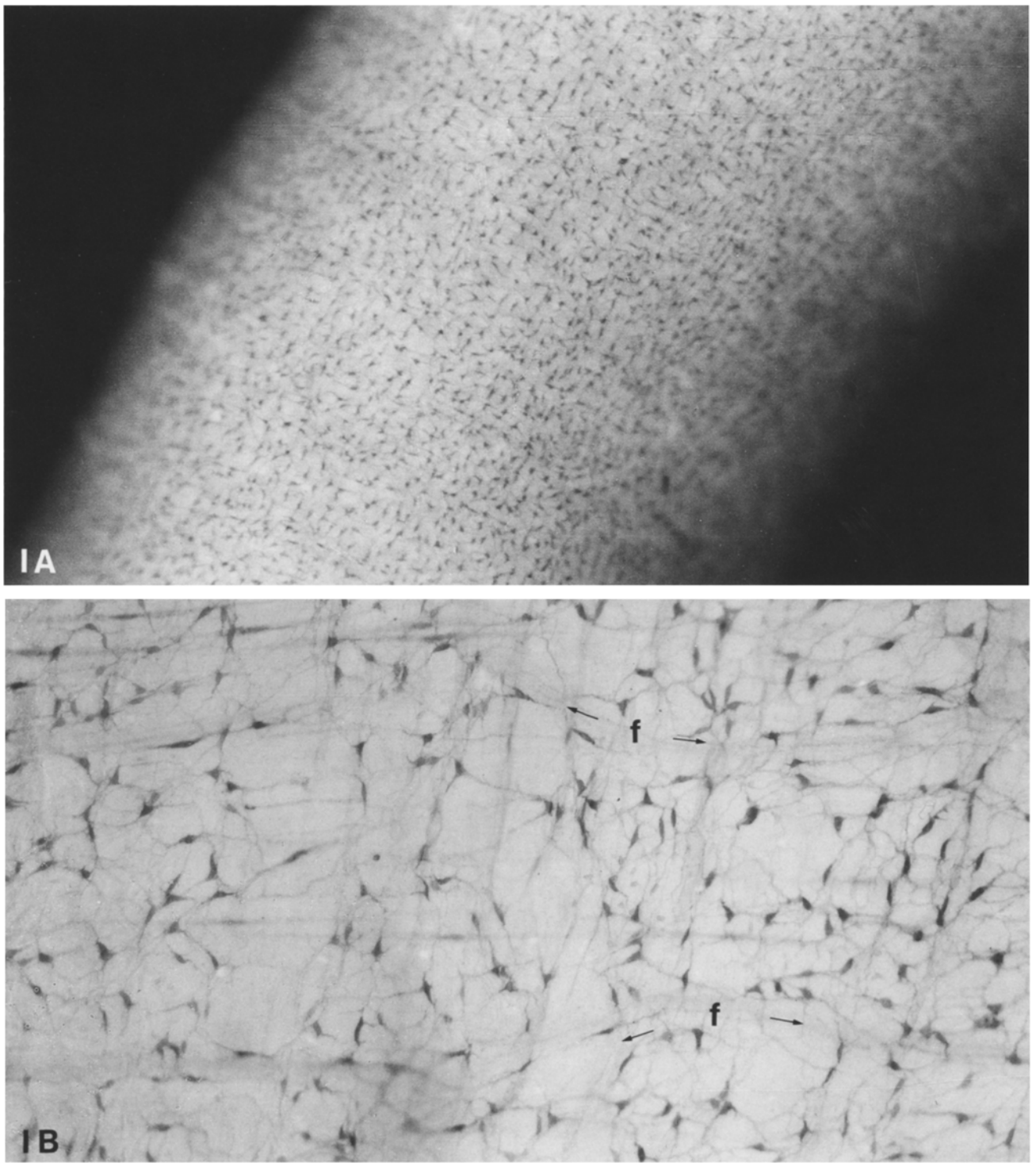


**Fig. 1 A, B.** Small intestine, stained with methylene blue, after lysolecithin treatment. **A** Suckling mouse (age, 5 days), jejunum. A cellular network of ICC-AP is visualized throughout the entire intestine. ×90. **B** Guinea pig, jejunum. Whole-mount of muscularis externa showing network of ICC, which is partly following the contours of fascicles (*f*). ×200

FITC-dextran 7 h (suckling mice) or from 1 to 5 days (adult animals) before sacrifice as previously described (Mikkelsen et al. 1985). Suckling mice were injected intraperitoneally with 0.1 ml 0.285 mM FITC-dextran, adult mice and guinea pigs received 0.2 ml intravenously and 1.0 ml intraperitoneally respectively, of 1.42 mM FITC-dextran in 1.54 mM NaCl.

Whole intestine was photographed with a Wild M 8 stereo microscope; fibre optic light guides were used for illumination. The whole-mounts were studied and photographed with a Leitz Orthoplan microscope. A HBO 200 W lamp was used to provide incident light for fluorescence microscopy with application of a Leitz ploemopak 2 equipped with the relevant Leitz filter blocks.

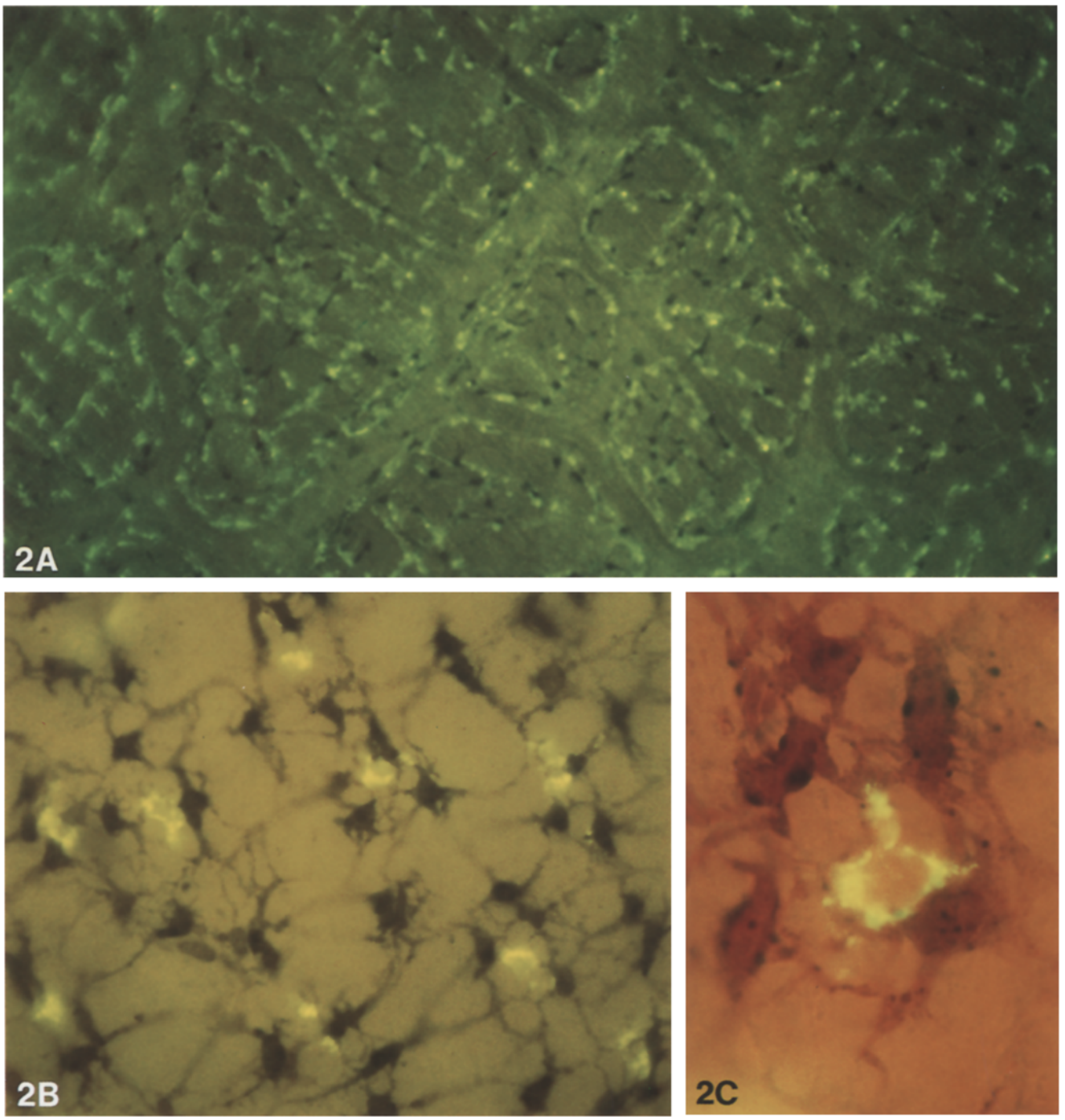


**Fig. 2A–C.** Whole-mounts of intestinal wall, double-labeling technique: FITC-dextran uptake by MLC and methylene blue staining of ICC after lysolecithin. **A** Guinea pig duodenum. ×100; **B** Adult mouse ileum. ×400; **C** Adult mouse duodenum. ×900

## Results

*Light microscopy*

With the small intestine from the suckling mouse a *brief* immersion in lysolecithin before methylene blue was optimal for staining of the entire network of ICC associated with Auerbach's plexus (ICC-AP). The effect of the lysolecithin treatment is shown in Fig. 1A; the staining was selective or nearly selective with some axons co-stained. The methylene blue stained the ICC-AP intensely dark blue showing both the primary and the secondary processes, which created a finely meshed cellular plexus. The density of cells appeared higher in the duodenum than in the ileum.

In adult mice, rats and guinea pigs the entire ICC-AP network was apparent as well (Fig. 1B). The branching pattern appeared more elaborate in mouse than in rat and guinea pig.

*Fluorescence microscopy*

In animals which had been injected with FITC-dextran, 7 h (suckling) or from 1 to 5 days before sacrifice, MLC-AP were observed as a constant cellular population with a strikingly regular distribution. In double-stained preparations it was apparent that the fluorescent MLC-AP were in close contact with ICC-AP over large membrane areas (Fig. 2A, 2B). Especially in the mouse the MLC-AP were always surrounded by ICC-processes (Fig. 2C). In guinea pig most MLC showed a similar relationship to ICC-AP.

## Discussion

Supravital methylene blue methods are notoriously difficult to standardize, the results varying even under apparently controlled circumstances (controlled aeration, pH, temperature, handling of tissue). It has previously been shown that through careful handling of the small intestine during isolation it is possible to restrict the uptake of dye to ICC-AP, single axons and a few contracted smooth muscle cells (Thuneberg 1982). However, even when the method is working in this relatively selective way the results are extremely variable with respect to completeness of staining: although ICC-AP form a continuous network along the small intestine the staining is usually restricted to smaller areas of this network.

By preincubation with lysolecithin (for review see Weltzier 1979) we have succeeded in obtaining a complete staining of the ICC-AP throughout the small intestine. Lysolecithin causes instantaneous lysis of the exposed cells at the surface of the intestine, i.e., following a short incubation only the mesothelial cells are affected. Observation of the mesothelium after a few seconds of exposure to lysolecithin reveals that the nuclear appearance is grossly changed. The optimal staining with methylene blue is obtained after a further incubation for one hour without lysolecithin in standard medium. Probably the primary effect on the mesothelium is limited to the accessible external plasma membrane and the drastic changes in tissue permeability occur only after further disintegration of the mesothelial layer. Since the selective staining of ICC with methylene blue depends on the preservation of the integrity of cells, it is likely that the effect of lysolecithin is restricted to the superficial layer.

A large number of seemingly specific modifications of methylene blue methods have been devised (for review, see Arthur and Shelley 1959), involving the addition of various chemicals, which have been found empirically to improve the results. Tested by other investigators the same substances have been without visible effect (Richardson 1969). Most likely permeability problems, varying from one tissue or organ to another, underlie many of the reported apparent contradictions. Such problems are inherent in many histological techniques. For instance, treatment of the intestine with detergent (Triton X-100) changed the demonstration of NADH-diaphorase activity from a less consistent and patchy distribution to a complete staining of myenteric neurons (Gabella 1987).

We have previously demonstrated (Mikkelsen et al. 1985) a regular and constant arrangement of MLC-AP in the small intestine. By electron microscopy the MLC-AP are closely enveloped by processes of ICC-AP.

By our double-labeling technique it is possible to demonstrate not only the arrangement of each cell type, but at the same time to show that the interrelation between ICC and MLC is a general feature of the organization of the two cell types.

While there is growing evidence in favour of a pacemaker function of ICC-AP, the role of the MLC is unknown. Ultrastructurally, the cells resemble macrophages, they possess the common macrophage surface antigens M1/70 and F4/80, but in contrast to peritoneal macrophages the MLC-AP all express Ia antigen (Mikkelsen et al. 1988) which belongs to the major histocompatibility complex and is believed to participate in the presentation of antigen to T lymphocytes (Hurley et al. 1983; Unanue 1984). However, their phagocytic properties appear limited (Mikkelsen et al. 1985), and a function as antigen presenting cells is difficult to reconcile with the fact that we never observe lymphocytes in or between the muscle layers. A third possibility (Thuneberg 1982), that MLC-AP are primarily secretory cells, is still speculative.

Current electrophysiological studies (Durdle et al. 1983; Hara et al. 1986; Suzuki et al. 1986; Smith et al. 1987) appear to limit the sites of origin of slow-wave activity in small and large intestine to those locations, where ICC are demonstrated by morphological studies. It is a major purpose of our study to call attention to the presence in one of these locations of another cell population, the MLC, and to encourage further studies of the cellular association between ICC and MLC.

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