

# Immunohistowax processing, a new fixation and embedding method for light microscopy, which preserves antigen immunoreactivity and morphological structures: visualisation of dendritic cells in peripheral organs

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## Abstract

**Aims**—To describe a new fixation and embedding method for tissue samples, immunohistowax processing, which preserves both morphology and antigen immunoreactivity, and to use this technique to investigate the role of dendritic cells in the immune response in peripheral tissues.

**Methods**—This technique was used to stain a population of specialised antigen presenting cells (dendritic cells) that have the unique capacity to sensitise naive T cells, and therefore to induce primary immune responses. The numbers of dendritic cells in peripheral organs of mice either untreated or injected with live *Escherichia coli* were compared.

**Results**—Numbers of dendritic cells were greatly decreased in heart, kidney, and intestine after the inoculation of bacteria. The numbers of dendritic cells in the lung did not seem to be affected by the injection of *E coli*. However, staining of lung sections revealed that some monocyte like cells acquired morphological and phenotypic features of dendritic cells, and migrated into blood vessels.

**Conclusions**—These observations suggest that the injection of bacteria induces the activation of dendritic cells in peripheral organs, where they play the role of sentinels, and/or their movement into lymphoid organs, where T cell priming is likely to occur.

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Keywords: dendritic cell; *Escherichia coli*; immunohistochemistry

The immune response is the result of multiple interactions between discrete cell populations of the immune system. Specialised cells (antigen presenting cells (APCs)) present the antigen, in the form of peptides in the groove of major histocompatibility complex (MHC) molecules, to helper T cells, which in turn activate the differentiation of effector cells, such as B cells or cytotoxic effector cells. APCs are widely distributed in the body, in lymphoid and

non-lymphoid organs,<sup>1</sup> whereas T cells recirculate through the lymphoid organs, where they are located in discrete sites. There is evidence that T cell priming occurs in the T cell zones of lymphoid organs.<sup>2,3</sup> Therefore, the first step of the immune response is likely to involve the migration of APCs and their redistribution into T cell areas.

The population of APCs is heterogeneous and includes dendritic cells, B cells, and macrophages. Among these cells, dendritic cells appear to have the unique capacity to sensitise naive T cells and are the APCs of the primary immune response.

The aim of our study was to analyse the movement of dendritic cells in peripheral solid organs of mice injected with Gram negative bacteria. To achieve this goal, a new immunohistochemistry processing (immunohistowax processing) method, based on a proprietary fixation and embedding medium, was developed and was shown to preserve both morphology and antigen immunoreactivity. In this processing, protein denaturation in the sample is minimised by the combined use of an aldehyde free zinc salt solution and a new embedding wax (Immunohistowax), which is liquid at low temperature (37°C). This approach allowed us to identify dendritic cells by morphological as well as phenotypic criteria. Our data show that most dendritic cells disappear from kidney, heart, and intestine after the injection of bacteria, whereas some lung dendritic cells become activated under the same conditions.

## Materials and methods

### ANIMALS

Female Balb/c and C57BL/6 mice were purchased from IFFA-CREDO (Brussels, Belgium) and maintained in our pathogen free facility.

### ESCHERICHIA COLI INOCULATION

The K504 *E coli* strain was kindly provided by Dr E Van Driessche (Laboratorium voor Chemie der Proteïnen, Vrije Universiteit Brussel, Belgium). Bacteria were grown in LB medium overnight at 37°C. Bacteria were pelleted by centrifugation at 3000 ×g for 10

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Table 1 Effect of zinc fixation on antigen preservation

Fixation	Dehydration	Staining score						
		MHC II	CD11c	B220	Mac-1	CD3	CD4	CD8
Zinc fixative (3 days)	Acetone	+++	+++	+++	+++	-	-	-
	Acetone	+++	+++	+++	+++	++	++	++
Zinc fixative (3 days)	Ethanol	+++	+++	+++	+++	++	++	++

Acetone: one bath of 100% acetone for six hours.

Ethanol: graded series of 30%, 50%, 70%, 90%, and 100% of ethanol, 30 minutes in each bath.

-, Negative; +, weakly positive; ++, positive; +++, strongly positive.

MHC, major histocompatibility complex.

minutes, washed twice in sterile phosphate buffered saline (PBS) and resuspended at  $5 \times 10^8$  bacteria/ml in PBS. A standard of absorbencies based on known colony forming units (CFU) was used to calculate the inoculum concentration. Mice received an intravenous injection of 200  $\mu$ l of *E coli* suspension. An *E coli* dose of  $10^8$  CFU was used in all experiments.

#### IMMUNOHISTOWAX PROCESSING

The tissue sample was fixed in a formaldehyde free zinc fixative (Immunohistofix; Intertiles, Brussels, Belgium) for three days at 4°C. Sample thickness did not exceed 5 mm to allow optimal infiltration of fixative and dehydrating agents. Dehydration was performed according

to two different protocols: the samples were either dehydrated in a graded series of ethanol baths: 30%, 50%, 70%, 90%, and 100% for 30 minutes each at room temperature, or samples were dehydrated in 100% acetone for six hours. The first protocol seems to preserve tissue morphology more effectively, whereas the second possibly preserved some antigens more efficiently. Infiltration was performed at 37°C by means of three baths of Immunohistowax for 20 minutes each. Tissue specimens were then embedded in Immunohistowax and mounted on wooden blocks. Blocks were stored at 4°C for at least one night before sectioning, and could be kept for up to six months at room temperature. Blocks can become difficult to cut at temperatures above 22°C, and were therefore kept at 4°C and cut promptly. Sections of 3–5  $\mu$ m were performed with a sliding microtome and individual sections were transferred directly using thin grids on a drop of water on gelatin precoated slides. Floating on a water bath is unsuitable because of the slight hydrophilicity of the wax. Slides were air dried at room temperature and stored at room temperature for up to several months.

#### IMMUNOSTAINING

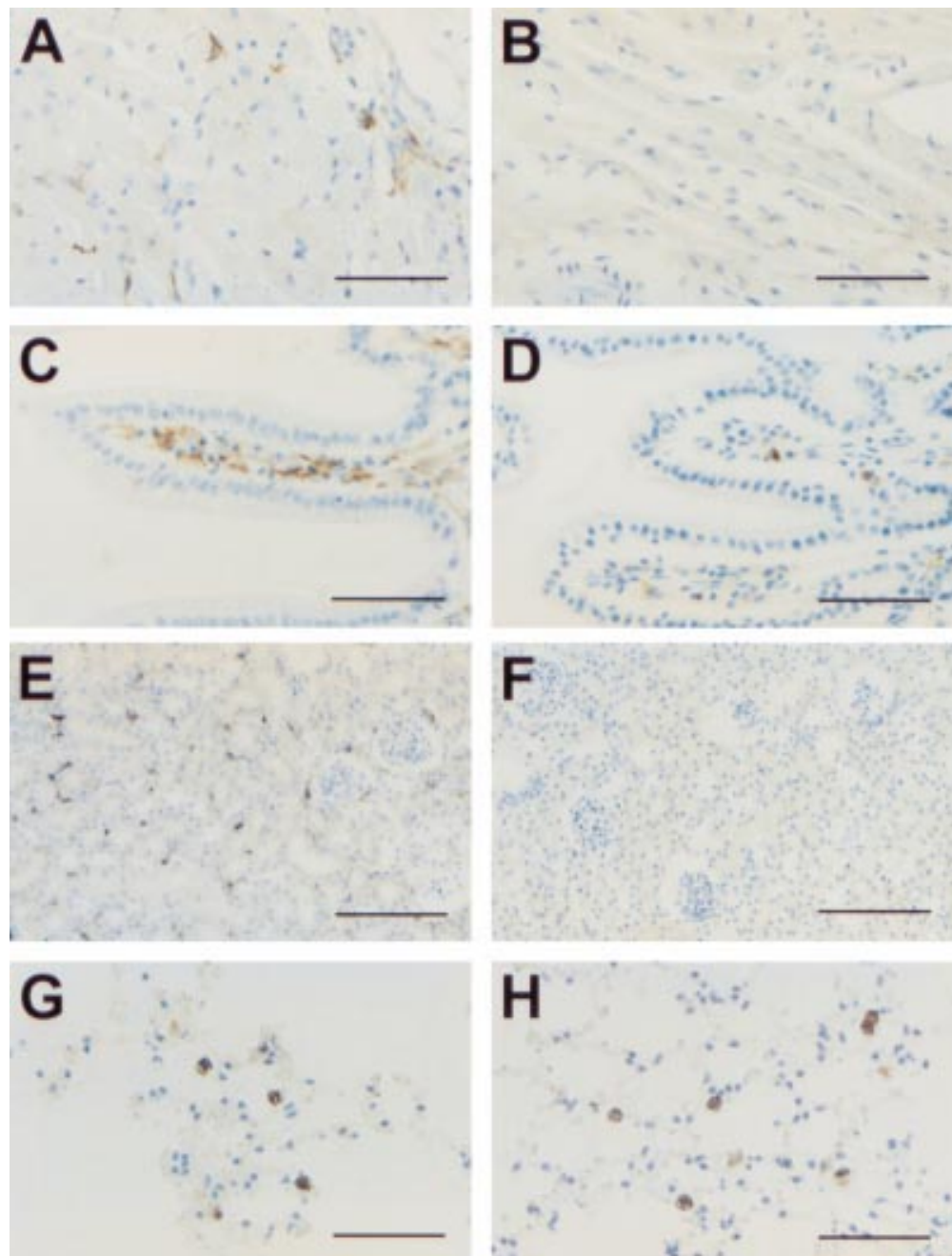
Immunohistowax processed sections were dewaxed in acetone for one to five minutes and

Table 2 Antigens successfully detected on Immunohistowax processed mouse tissue sections

Antigens	Clones	Specificity	Dilution	Sources
CD2	12-15	Cell adhesion molecule	10–20 $\mu$ g/ml	Southern Biotechnology Associates, Inc
CD3	7D6	T cells	20–30 $\mu$ g/ml	Coulie and colleagues <sup>14</sup>
CD4	RM4-5	T helper cells	20 $\mu$ g/ml	Pharmingen
CD8	53-6.7	T cells	20 $\mu$ g/ml	Pharmingen
CD90/Thy 1.2	53-2.1	T cells	10–20 $\mu$ g/ml	Pharmingen
TCR $\alpha$ $\beta$	H57-597	T cells (TCR)	20–30 $\mu$ g/ml	Pharmingen
TCR $\gamma$ $\delta$	13D5	T cells (TCR)	20–30 $\mu$ g/ml	Peterman and colleagues <sup>15</sup>
TCR V $\beta$ 8	F23.2	T cells (TCR)	20 $\mu$ g/ml	ATCC
TCR V $\beta$ 6	44.22.1	T cells (TCR)	20 $\mu$ g/ml	Payne and colleagues <sup>16</sup>
B220/CD45R	RA3-6B2	B cells	5–10 $\mu$ g/ml	Pharmingen
IgD	LoMD6	B cells	5–10 $\mu$ g/ml	Lefebvre and colleagues <sup>17</sup>
IgM	LoMM9	B cells	5–10 $\mu$ g/ml	Lefebvre and colleagues <sup>17</sup>
MHC-II	14.4.4	B cells, DC, M $\phi$	5–10 $\mu$ g/ml	ATCC
CD11c	N418	Mainly DC	5–10 $\mu$ g/ml	Metlay and colleagues <sup>18</sup>
CD11c	HL3	Mainly DC	5–10 $\mu$ g/ml	Pharmingen
CD11b/Mac-1	M1/70	M $\phi$	5–10 $\mu$ g/ml	Pharmingen
NK	5E6	NK cells	10–20 $\mu$ g/ml	Pharmingen
Pan-NK	DX5	NK cells	10–20 $\mu$ g/ml	Pharmingen
Ly-6G/GR1	RB6-8C5	Granulocytes	10–20 $\mu$ g/ml	Pharmingen
PMN antigen	7/4	Neutrophils	10–20 $\mu$ g/ml	Caltag
CD40	3/23	B cells, DC, M $\phi$	10–20 $\mu$ g/ml	Hasbold and colleagues <sup>19</sup>
Intracytoplasmic granules	2A1	Mature DC	5–10 $\mu$ g/ml	Inaba and colleagues <sup>20</sup>
Intracytoplasmic granules	M342	Mature DC	Undiluted supernatant	Agger and colleagues <sup>21</sup>
DEC-205	NLDC-145	Mature DC	20–30 $\mu$ g/ml	Kraal and colleagues <sup>22</sup>
CD35	8C12	CR1 (Follicular DC)	5–10 $\mu$ g/ml	Pharmingen
CD86/B7.2	GL1	Activated B cells, DC, M $\phi$	10–20 $\mu$ g/ml	Hathcock and colleagues <sup>23</sup>
CD25	7D4	Activated T cells, B cells, DC	10–20 $\mu$ g/ml	Pharmingen
Ly-77	GL7	Activated T cells, B cells	10–20 $\mu$ g/ml	Pharmingen
CD44	1M7	Activated T cells	10–20 $\mu$ g/ml	Pharmingen
CD69	H1.2F3	Activated T cells	10–20 $\mu$ g/ml	Pharmingen
Phosphotyrosin	4G10	Activated cells	20–30 $\mu$ g/ml	Upstate Biotechnology Corporation
OX40	mOX40L-hIgG1*	Activated T cells	20–30 $\mu$ g/ml	Cantab Pharmaceutical Research Ltd
VCAM-1	M/K-2	Adhesion molecule	10–20 $\mu$ g/ml	R&D Systems
Anti-Ars Ig	Ars-BSA <sup>o</sup>	Ars specific B cells	20 $\mu$ g/ml	
IL-2	S4B6	Cytokine	20–30 $\mu$ g/ml	Pharmingen
IL-4	11B11	Cytokine	20–30 $\mu$ g/ml	Pharmingen
INF- $\gamma$	Db1	Cytokine	20–30 $\mu$ g/ml	Paineau and colleagues <sup>24</sup>
IL-10	JESS-2A5	Cytokine	20–30 $\mu$ g/ml	Pharmingen

\*Fusion protein; <sup>o</sup>synthesised hapten carrier protein.

Ars, arsenate; BSA, bovine serum albumin; DC, dendritic cell; h, human; IFN- $\gamma$ , interferon  $\gamma$ ; Ig, immunoglobulin; IL, interleukin; L, ligand; MHC, major histocompatibility complex; M $\phi$ , macrophage; NK, natural killer; PMN, polymorphonuclear cell; TCR, T cell receptor; VCAM, vascular cell adhesion molecule.



**Figure 1** Immunostaining of major histocompatibility complex (MHC) class II positive or CD11c positive cells in organs of mice injected or not injected with bacteria. Heart (A and B), intestine (C and D), kidney (E and F), and lung (G and H). Sections from Balb/c mice injected 24 hours previously with phosphate buffered saline (A, C, E, and G) or *Escherichia coli* (B, D, F, and H) were stained with anti-I-E<sup>d</sup> (A–F) or anti-CD11c (G and H) monoclonal antibodies, and further counterstained with haemalun. Scale bar, 50 μm (A, B, C, D, G, and H), 100 μm (E and F).

transferred to PBS. Immunohistochemical staining was performed as follows.

#### *Inhibition of endogenous peroxidases*

If peroxidase was used for visualisation, the slides were first treated with 3% H<sub>2</sub>O<sub>2</sub> in PBS for 30 to 60 minutes to block endogenous peroxidase; the use of methanol was avoided because it might be detrimental for certain antigens, such as T cell markers.

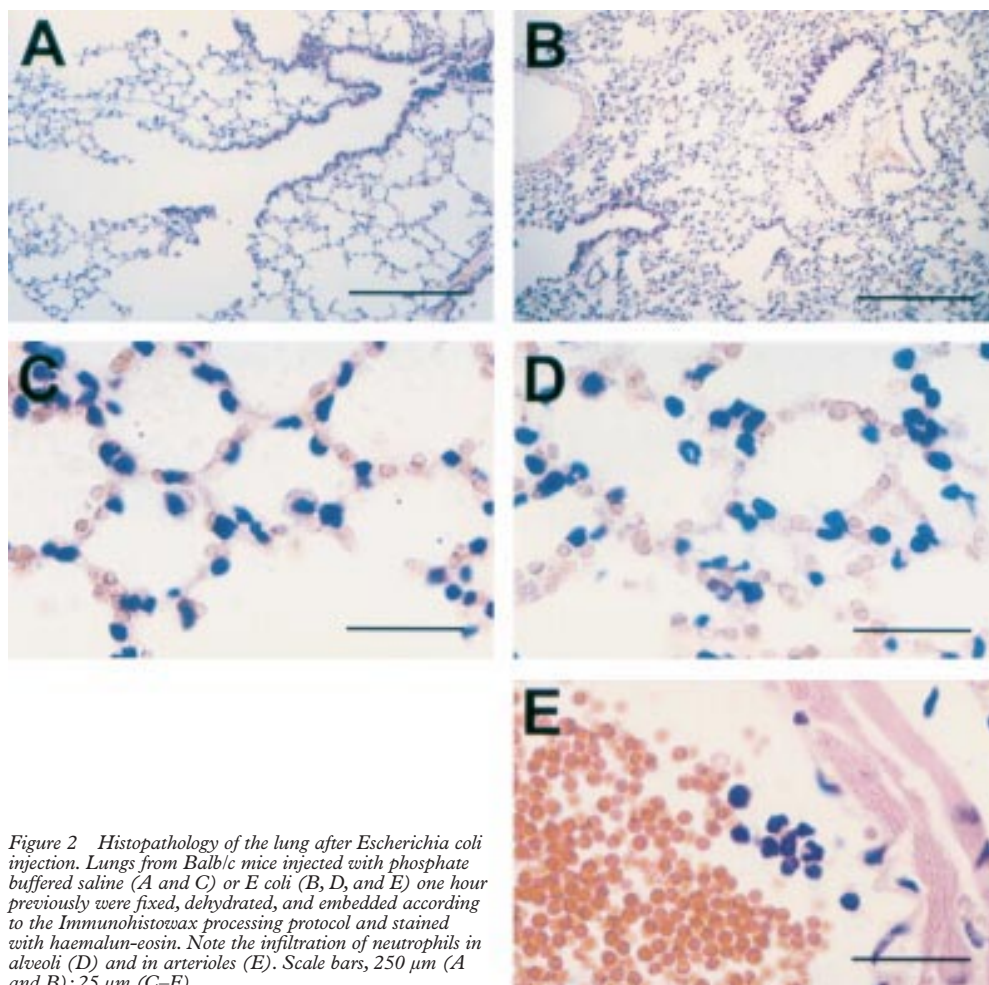
#### *Saturation step*

We commonly use the blocking reagent (catalogue number, 1096176) from Boehringer (Brussels, Belgium; 1% in PBS (PBS-BR)) to

saturate non-specific reaction sites because it gives a lower background than bovine serum albumin, horse serum, or goat serum. Sections were incubated in PBS-BR at room temperature for 30 minutes.

#### *Single staining*

Sections were washed in PBS and incubated with antibodies (5–25 μg/ml) for one to three hours at room temperature or overnight at 4°C in PBS-BR. Primary monoclonal antibodies gave better results when coupled to biotin or fluorescein isothiocyanate (FITC). Biotinylated antibodies were visualised with ABC kits from Vector Laboratories (Burlingame,



**Figure 2** Histopathology of the lung after *Escherichia coli* injection. Lungs from Balb/c mice injected with phosphate buffered saline (A and C) or *E coli* (B, D, and E) one hour previously were fixed, dehydrated, and embedded according to the Immunohistowax processing protocol and stained with haemalun-eosin. Note the infiltration of neutrophils in alveoli (D) and in arterioles (E). Scale bars, 250 µm (A and B); 25 µm (C–E).

California, USA; 1/100 in PBS-BR) for 30 minutes at room temperature. Peroxidase was revealed using either diaminobenzidine substrates with or without metal enhancer (Sigma, Bornem, Belgium), TMB, Vector SG Substrate Kit, Vector VIP Substrate Kit (Vector), or AEC (Sigma). Substrate kits from Vector Laboratories for alkaline phosphatase were used according to the manufacturer's recommendations. FITC conjugated antibodies were visualised by incubation for 30 minutes with anti-FITC alkaline phosphatase or peroxidase Fab fragment (Boehringer), diluted at 1/500 in PBS-BR.

#### Counterstaining

Single immunostained sections were counterstained with haematoxylin or methyl green depending on the substrate colour.

When double or triple staining was performed with biotinylated antibodies, excess biotin from the first antibody was blocked with the Vector blocking kit. In the case of multiple peroxidase stainings, enzymatic activity linked to the first antibody was neutralised by incubating sections in H<sub>2</sub>O<sub>2</sub> (3% in PBS) for 20–30 minutes.

#### Mounting

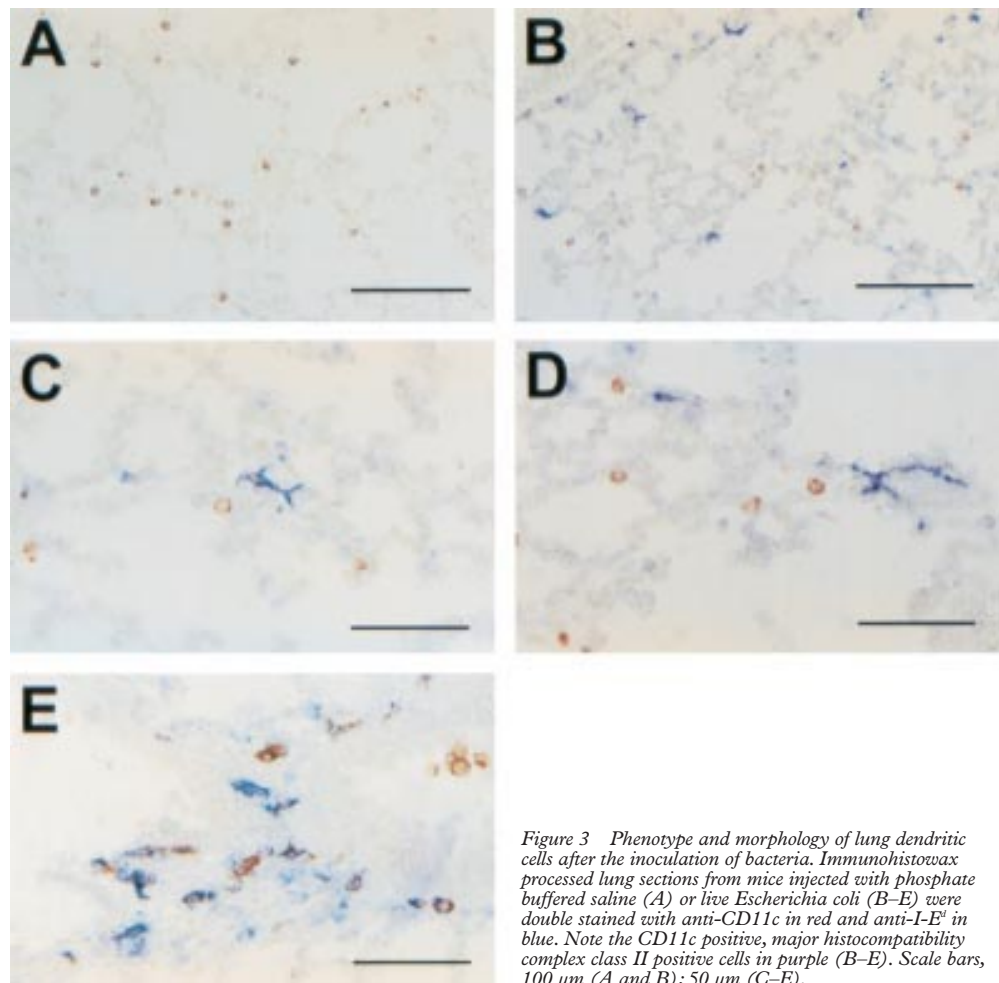
Slides were mounted in Aquatex (Merck, Overijse, Belgium) or Polymount (Polysciences, Warrington, USA) depending on the solubility of substrates.

## Results

### IMMUNOHISTOWAX PROCESSING

Morphological observations require thin tissue sections, which are best obtained after the infiltration and embedding of specimens with wax or plastic. However, because waxes are poorly soluble in water, their use requires pretreatment with fixatives and dehydrating solvents, which is known to affect protein structure. In addition, fixation and/or embedding can also affect the immunodetection of many cellular markers as a result of protein denaturation caused by chemical and/or thermal injury.<sup>4–6</sup> Although we have developed a novel wax that is liquid at near physiological temperature (37°C), its poor solubility in water required tissue dehydration before embedding. Dehydration was performed with acetone or ethanol, which were fully miscible in the liquid wax. Unfortunately, and as expected from previous reports,<sup>7</sup> the dehydration step affected the staining of many antigens. The data in table 1 show that dehydration prevented the staining of CD3, CD4, and CD8 antigens. By contrast, other antigens, such as MHC class II, CD11c, B220, and Mac-1 were not altered by dehydration with one bath of 100% acetone for six hours.

Because exposure to organic solvents before embedding could not be avoided, we attempted to protect antigenic structures from chemical denaturation by increasing protein stability



**Figure 3** Phenotype and morphology of lung dendritic cells after the inoculation of bacteria. Immunohistochemical processed lung sections from mice injected with phosphate buffered saline (A) or live *Escherichia coli* (B–E) were double stained with anti-CD11c in red and anti-I-E<sup>d</sup> in blue. Note the CD11c positive, major histocompatibility complex class II positive cells in purple (B–E). Scale bars, 100  $\mu$ m (A and B); 50  $\mu$ m (C–E).

before fixation. Engineered metal chelation in proteins has been used successfully to stabilise proteins against denaturation.<sup>8,9</sup> We choose  $Zn^{2+}$  as a metal ion, based on its known ability to interact with at least four amino acids (primarily histidine, and to a lesser extent, aspartic acid, glutamic acid, and cysteine) through binding to nitrogen, oxygen, and sulphur atoms.<sup>10,11</sup> Its thermodynamic properties have been shown to promote favourable entropic effects, which enhance the stability of secondary protein structure.<sup>12</sup> Ligand motifs that can be used for metal binding are His-XXX-His for an  $\alpha$ -helix, His-X-His for a  $\beta$ -strand, and His-XX-His for a reverse type II  $\beta$ -strand.<sup>13</sup> In some cases, one or two histidine(s) might be replaced by aspartate or cysteine. We analysed mouse protein sequences and found that at least one motif was present in all sequences investigated, the mean value being 3.5 motifs/sequence. Although structural data are not available for most antigenic proteins, we assumed that a sufficient number of these motifs was indeed able to bind a metal ion.

Tissue specimens were pretreated with a zinc fixative (Immunohistofix), dehydrated, and embedded in Immunohistowax. We compared the staining of several antigens on tissue samples, pretreated or not with zinc fixative. The data in table 1 show that the staining of CD3, CD4, and CD8 antigens was preserved by pretreatment with the zinc fixative. We fur-

ther tested several monoclonal or polyclonal antibodies, fusion proteins, lectins, and enzymes for the staining of membrane or intracellular proteins, carbohydrate residues, surface receptors, and apoptotic cells. The results in table 2 show that a large number of molecules expressed by T or B cells, NK cells, macrophages, and dendritic cells could be detected on Immunohistowax processed sections. In particular, determinants expressed upon cell activation were stained using monoclonal antibodies (specific for CD25, CD44, CD69, CD86, Ly-77, or DEC-205) or a fusion protein (OX40 ligand-human IgG1). B cells specific for the hapten arsonate were visualised on spleen sections using the hapten coupled to bovine serum albumin (BSA), whereas T cells were stained with a monoclonal antibody specific for their antigenic receptor. Interleukin 2 (IL-2), IL-4, interferon  $\gamma$  (IFN- $\gamma$ ), and IL-10 were detected in the cytoplasm of activated T cells in situ, using double staining with antibodies to T cells and to lymphokines. Using this processing method, we previously identified apoptotic cells in tissues by double staining with antibodies to cell surface markers and the TUNEL (TdT mediated dUTP nick end labelling) reaction.<sup>25</sup> It should be noted that, among all presently tested antibodies, only two did not work with this technique: 2C11 (hamster antimouse CD3) and GK1.5 (rat antimouse CD4).

#### INJECTION OF GRAM NEGATIVE BACTERIA REDUCES THE NUMBER OF DENDRITIC CELLS IN KIDNEY, HEART, AND INTESTINE

Dendritic cells are a trace population in most organs, usually display dendrites, and express selected surface markers, such as MHC class II molecules and/or CD11c. We stained sections of various organs with CD11c or MHC class II specific monoclonal antibodies. Dendritic cells in the kidney, heart, and intestine were found to be negative for CD11c expression. In the heart, most class II positive cells with a dendritic morphology (presumably dendritic cells) are located in the pericardium (fig 1A), whereas few cells were detected in the myocardium (not shown). Dendritic cells in both sites decreased in numbers after the injection of *E coli* (fig 1B and data not shown). In the intestine, class II positive cells were detected in the connective tissue (lamina propria) of the villi (fig 1C). The injection of live bacteria resulted in the loss of most MHC class II positive cells (fig 1D). As shown in fig 1E, MHC class II positive cells with a typical dendritic morphology could be found in the renal cortex around the glomeruli in the kidney of PBS treated mice. Intravenous inoculation of *E coli* led to a reduction in the number of MHC class II positive cells (fig 1F).

#### INJECTION OF *E COLI* INDUCES PHENOTYPIC AND MORPHOLOGICAL CHANGES IN LUNG DENDRITIC CELLS AND THEIR MOVEMENT TO BLOOD VESSELS

By contrast, the numbers of CD11c positive cells in lung remained unchanged after the inoculation of live *E coli* (compare fig 1G and 1H). Staining of lung sections with haemalun-eosin clearly shows constriction of the alveoli (fig 2A and B) and infiltration of the lung parenchyma by neutrophils (fig 2D and E), starting one hour after the inoculation of live *E coli*. In addition, immunostaining of sections revealed CD11c positive cells with a globular shape within the alveoli (fig 3A). These cells did not express MHC class II molecules (fig 3A) and could be alveolar macrophages or dendritic cells at a very immature stage (see below). Surprisingly, some of these CD11c positive cells (approximately 20–25%) increased in size, acquired a dendritic morphology, and upregulated the expression of I-E molecules (fig 3B–E) as early as one hour after the injection of live bacteria. Later on (one to six hours after treatment), these dendritic like, CD11c positive, MHC class II positive cells seem to migrate to the blood vessels (fig 3C and D).

#### Discussion

Our report describes a new fixation and embedding technique, called Immunohistowax processing, that permits immunostaining of a large variety of antigens and preserves the morphology of all tissues tested. To achieve minimal denaturation of proteins during dehydration and embedding, the protein structures were stabilised by pretreatment with an aldehyde free zinc fixative before dehydration with ethanol or acetone. Tissue specimens were

then embedded in an inert wax at 37°C. This processing allowed antigen immunodetection and morphological analysis of thin sections (3–5 µm).

The Immunohistowax processing technique allowed us to detect a trace population of APCs—cells of the dendritic family—in peripheral organs using phenotypic and morphological criteria.

It is generally believed that dendritic cells play the role of sentinels in the periphery, and upon the encounter of an appropriate signal (signal of danger?) are redistributed to T cell areas, where they probably prime T cells.<sup>26</sup> The major role of dendritic cells in inducing primary immune responses correlates with some specialisation of function over time and space. In non-lymphoid tissues, dendritic cells are present in an immature state: well equipped to capture and process antigens but unable to sensitise T cells optimally. In secondary lymphoid organs, dendritic cells are mature; that is, they poorly capture and process proteins but have the capacity to prime naive T cells. The data presented here indicate that the inoculation of live bacteria induces the disappearance of most dendritic cells from the heart, kidney, and intestine. Their loss could be attributed to migration from these organs or death by apoptosis. We and others have shown previously that the injection of lipopolysaccharide or toxoplasma extracts provoked the migration of splenic dendritic cells from the marginal zone between the red and white pulp to the areas where T cells are located.<sup>27–28</sup> These observations, together with those of Roake *et al*,<sup>29</sup> suggest that dendritic cells that have encountered bacteria might migrate to T cell zones in lymph nodes. Experiments are under way to test whether new migrant cells, bearing microbial antigens, can be detected in draining lymph nodes.

In the pulmonary alveoli, some CD11c positive cells acquire a dendritic morphology, upregulate the expression of MHC class II molecules, and migrate into proximal capillaries. These observations are reminiscent of a report by Randolph *et al*,<sup>30</sup> showing that monocytes differentiate into dendritic cells in vitro, after migration across the endothelium in the subluminal to luminal direction, a phenomenon that is potentiated by an additional stimulus, such as lipopolysaccharide of zymosan particles. It is therefore tempting to speculate that, in the lung, MHC class II negative, CD11c positive monocytes are induced to differentiate into dendritic cells through cross-endothelial migration and exposure to microorganisms. Additional work will be required to test this hypothesis.

In conclusion, Immunohistowax processing seems to allow optimal conservation of morphology and immunoreactivity. Indeed, almost all antigens tested were detected in Immunohistowax processed sections, including antigens that were not stained in paraffin wax embedded sections or cryosections. Of note, this method only requires standard equipment and does not rely on the antigen retrieval technique. We believe that Immunohistowax

processing will be useful to identify *in situ* the cell populations that secrete various cytokines and to define more accurately the spatial and temporal organisation of the immune response.

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## Immunohistochemical processing, a new fixation and embedding method for light microscopy, which preserves antigen immunoreactivity and morphological structures: visualisation of dendritic cells in peripheral organs

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