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Histology and Histopathology

Cellular and Molecular Biology

Invited Review

The role of activated cytotoxic T cells in inflammatory bowel disease

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Summary. The role of cell-mediated cytotoxicity in the pathogenesis of ulcerative colitis and Crohn's disease has been controversial since reports indicating either a decreased, or an increased, activity of cytotoxic T cells in active stages of inflammatory bowel disease exist. Some of these discrepancies may be attributed to the fact that so far mostly peripheral blood lymphocytes rather than intestinal T cells have been examined. To overcome some of these limitations we performed in situ hybridizations for the detection of perforin and granzyme A mRNA expressing cells, i.e. of cytotoxic cells activated in situ, in the affected intestinal mucosa. These studies revealed increased frequencies of activated, cytotoxic T cells in active stages of ulcerative colitis and Crohn's disease. Interestingly, activated perforin mRNA expressing T cells are present both in the CD4 and in the CD8 T cell subsets. In the latter T cell subset up to 60% of the mucosal T cells isolated from the affected sites express perforin mRNA at detectable levels. The elevated frequency of activated cytotoxic cells and their histological distribution also in close proximity to the epithelial cells may thus indicate an important role for cytotoxic cells in the pathogenesis of inflammatory bowel disease since activated cytotoxic T cells may further exacerbate the inflammatory process through the production of pro-inflammatory cytokines such as interferon-y or tumor necrosis factor-a, but also through the release of pro-inflammatory cytokines and chemokines upon lysis of epithelial cells and the increased influx of luminal antigens at the site of epithelia1erosions.

Key words: Ulcerative colitis, Crohn's disease, Cell-mediated cytotoxicity, Perforin, Granzyme A

Abbreviations used: CD: Crohn's disease, IBD: inflammatory bowel disease, CTL: cytotoxic T lymphocytes, TNF: tumor necrosis factor, IEL: intraepithelial lymphocytes, UC: ulcerative colitis, IL: interleukin

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Introduction

Crohn's disease (CD) and ulcerative colitis (UC) represent the two major clinical entities of chronic relapsing inflammatory bowel disease (IBD). Despite increasing research activities etiologic agents or well-defined factors responsible for the initiation of the inflammatory response still remain enigmatic. In the past, several factors including familial disposition, psychosocial factors, dietary factors and bacterial or viral infections have been postulated to contribute to the initiation and/or perpetuation of the disease (Fig. 1). Our improved understanding of immunoregulatory mechanisms and the increased interest of the research community in mucosal immunity lead to a large number of studies focusing on the role of aberrant immune reactions in the pathogenesis of IBD. Consequently, in the past few years evidence for a crucial involvement of a dysregulated immune response in the intestinal mucosa in patients with IBD has accumulated.

Indications for immunopathological factors contributing to the pathogenesis of IBD

The occurrence of autoantibodies against perinuclear cytoplasmic antigens in neutrophils (pANCA) has been long considered as a main indication for the contribution of the immune system to the pathogenesis of UC. Although more recent studies demonstrated that pANCA represents the consequence of a mucosal immune response rather than the disease initiating event (Targan et al., 1995) in UC, indications for an involvement of the immune system in the pathogenesis of UC and CD have increased considerably over the past few years. Most compelling evidence for a contribution of a dysregulated immune response to the initiation and perpetuation of IBD has been obtained in several mouse models of colitis. Mice lacking specifically the gene encoding interleukin (IL)-2 due to homologous recombination of the endogenous gene locus with a disrupted, non-functional gene, spontaneously develop a colitis-like disease at an advanced stage of age when kept under conventional conditions of maintenance (Sadlack et al., 1993). The spontaneous development of a colitis in IL-2-

deficient mice is surprising since these mice are capable of mounting an almost normal humoral, and cell-mediated immune response. Similar observations of a spontaneous development of a colitis-like disease have been subsequently observed also in other mouse strains deficient for genes crucially involved in a specific immune response such as the gene encoding IL-10, transforming growth factor (TGF)-ß, T cell receptor α, or δ-chain, ß2-microglobulin or a G-protein. Evidence for a central role of T cells in the initiation of colitis has been obtained by the adoptive transfer of CD4+ CD45RBhi T cells, representing presumably naive CD4 T cells, into immune deficient scid mice that lack mature T and B cells (Morrissey et al., 1993; Powrie et al., 1993). Upon adoptive cell transfer, recipients start to lose weight concurrent with histopathological alterations resembling in many aspects UC in human patients (Morrissey et al., 1993; Powrie et al., 1993).

Based on a series of studies performed in these spontaneous mouse models of colitis several conclusions can be made that may also be relevant for the pathogenesis of IBD in humans. Severe immunoregulatory defects that have only a limited impact on the outcome of a normal humoral or cellular immune response, can lead to the development of a colitis. Different defects, such as a deficiency for a cytokine gene, MHC class I antigens or T cell receptor chain genes may lead to comparable histopathological alterations. For the development of the disease a microbial colonization is required since animals kept under strict germ-free conditions of maintenance do not develop the disease. Kinetics of disease onset and severity in these mouse models of colitis is also controlled by the overall genetic background since different mouse strains with the same gene deficiency (e.g. the IL-2 gene) show distinct incidence rates and severity of colitis. Taken together, observations made with these novel mouse models of colitis (e.g. gene-deficient mouse strains, transfer of naive CD4 T cell subsets into immune deficient mice) clearly demonstrate the requirement for a well balanced, tightly regulated immune system in the intestinal mucosa, particularly in the colon to maintain the tissue homeostasis in the bowel wall.

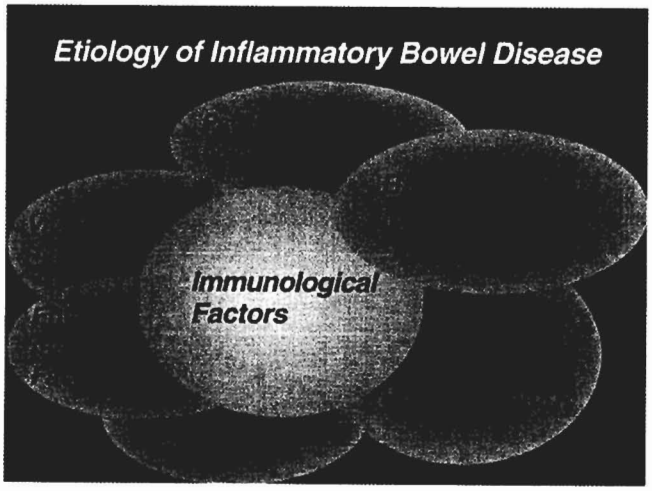

Fig. 1. Factors postulated to contribute to the pathogenesis of inflammatory bowel disease.

Role of activated cytotoxic cells in the pathogenesis of IBD

Reports on the role of activated cytotoxic T cells and NK cells in the pathogenesis of IBD have been controversial for some time. Indications for an increased (Okazaki et al., 1993; Simpson et al., 1995), but also for a decreased (Ginsburg et al., 1983; Egawa and Hiwatashi, 1986; Ruthlein et al., 1992), activity of cytotoxic cells have been reported. Part of the controversial results may be explained by the different sources of cytotoxic cells used for analysis, in particular, peripheral blood lymphocytes, and lymphocytes from the intestinal intraepithelial compartment and lymphocytes from the lamina propria, but also by the technical difficulties to quantitatively recover fully activated lymphocytes from the intestinal mucosa for subsequent functional analyses.

CD8 T cells are activated in situ in the intestinal mucosa in active IBD

Upon antigen-specific activation of cytotoxic T cells and NK cells, the genes for perforin and a family of serine proteases, also termed granzymes, are rapidly induced. Upon synthesis, perforin and granzymes are stored in the cytoplasmic granules of cytotoxic cells and are released in a polarized way into the intercellular space upon recognition of a target cell by the cytotoxic effector cell. Hence, detection of mRNA for perforin and granzymes by in situ hybridization has been widely used to follow activation of cytotoxic effector cells in the tissue and to indirectly assess the cytotoxic activity of tissue-infiltrating cells (Griffiths and Mueller, 1991). In a recent study we directly monitored the presence of recently activated, perforin, and granzyme A mRNA expressing cells in the intestinal mucosa of patients with CD, UC and of normal controls (Muller et al., 1998). As summarized in Figs. 2, 3, in the affected intestinal mucosa of patients with active IBD 4- to 6-fold increased frequencies of perforin or granzyme A mRNA-positive intraepithelial lymphocytes (IELs) (Fig. 2) and lamina propria cells (Fig. 3) are observed. Perforin mRNA expressing cells detected by in situ hybridization in the colonic mucosa from a patient with active UC, and from a normal control patient, are shown in Fig. 4. For the direct assessment of the frequency of activated cytotoxic cells among the two major T cell subsets, i.e. CD4, and CD8 T cells, intestinal T cells were sorted according to their surface phenotype on a FACSVantage® for subsequent in situ hybridization. This analysis confirmed that in affected intestinal mucosa the frequencies of activated cytotoxic cells is

greatly increased when compared with cells isolated from corresponding intestinal mucosa of normal controls, particularly among CD8, but to a lesser degree also in CD4 T cells. The observed frequencies of activated, cytotoxic perforin mRNA expressing CD8 T cells observed in active CD and UC are even higher than during peak activity of cell-mediated cytotoxicity during the rejection of an experimental allogeneic myocard graft in mice (Mueller et al., 1988) (Table 1). Frequencies of activated cytotoxic CD8 T cells isolated from affected intestinal areas were lower in patients with active UC than in patients with CD where in certain patients more than 60% of isolated CD8 T cells of the lamina propria expressed the perforin gene. Perforin mRNA-containing cells can be considered as being functionally active cytotoxic T lymphocytes (CTLs), activated in situ in the affected mucosa (Muller et al., 1998). When tissue samples with distinct histopathological signs of disease activity are compared for their frequency of perforin or granzyme A-mRNA expressing cells, no significant differences are found among the different stages of disease activity. Moreover, even in macroscopically unaffected mucosal area adjacent to affected areas in patients with CD elevated frequencies of activated cytotoxic cells expressing perforin mRNA are found, thus indicating that in CD aberrant mucosal immune reactions may not be restricted to sites with macroscopical signs of disease. At present it is unclear why the intestinal mucosa may show only minimal histopathological alterations despite the presence of elevated frequencies of activated CTLs. One may speculate that these activated CTLs represent migrating CTLs that have been activated antigen-specifically in the adjacent affected mucosa and

Table 1. Frequency of activated CD8 T cells.

Transplant (MHC class I/II mismatched myocard graft) - infiltrating CD8 T cells:
11.3% Granzyme A mRNA positive
14.2% Granzyme B mRNA positive
(Mueller et al., 1988)
Normal intestine (small and large intestine)
CD8 IEL: 6.7% Perforin mRNA positive
CD8 LPL: 9.4% Perforin mRNA positive
Ulcerative colitis
CD8 IEL: 18.2% Perforin mRNA positive
CD8 LPL: 17.5% Perforin mRNA positive
Crohn's disease
CD8 IEL: 24.3% Perforin mRNA positive
CD8 LPL: 36.1% Perforin mRNA positive
(Müller et al., 1998)

Frequencies of activated, perforin-mRNA expressing CD8 T cells in the affected intestinal mucosa of patients with active IBD exceed considerably the corresponding values obtained in the analysis of allotransplant-infiltrating CD8 T cells in a mouse model.

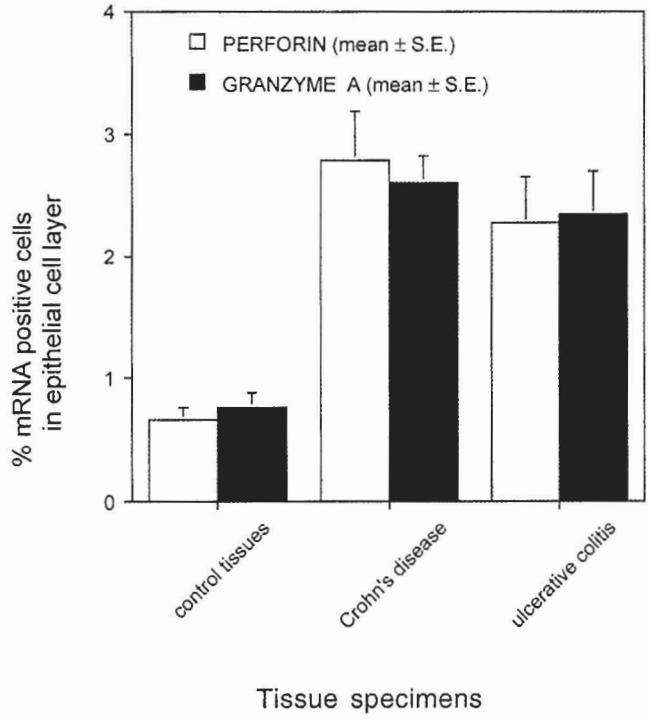

Fig. 2. Frequency of activated, granzyme A-, or perforin mRNA expressing cytotoxic cells in the intestinal epithelium as determined by in situ hybridization of tissue sections from patients with CD, UC and controls (adapted from Muller et al., 1998).

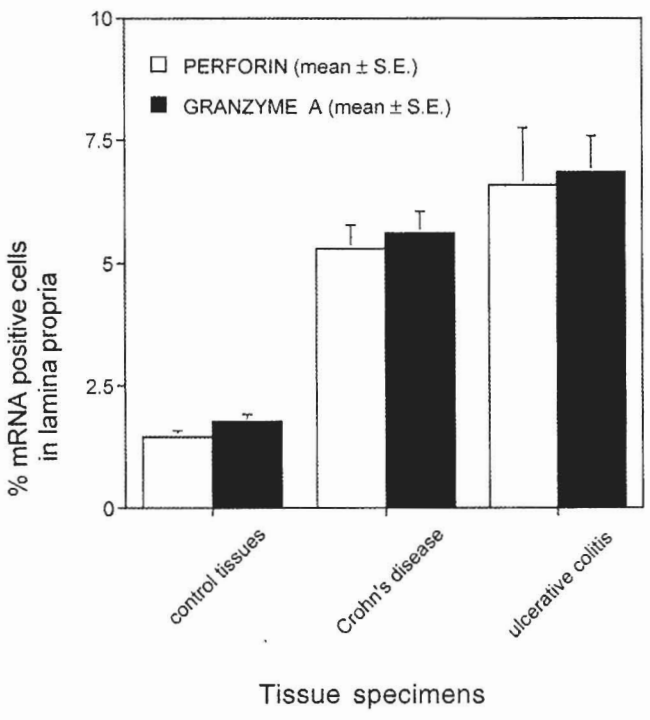

Fig. 3. Frequency of activated, granzyme A-, or perforin mRNA expressing cytotoxic cells in the intestinal lamina propria as determined by in situ hybridization of tissue sections from patients with CD, UC and controls (adapted from Muller et al., 1998).

subsequently migrate to other sites. At these sites, however, the CTL-triggering antigens may not be expressed; hence, pre-activated CTLs may not be induced to release their cytolytic proteins thus sparing these sites from deleterious CTL mediated cytolysis. Alternatively, enhanced expression of CD95L (FasL) on epithelial cells present in the unaffected mucosal areas may lead to the elimination of activated CTLs that upon activation also express CD95 (Fas) on their surface. This mechanism, mediating protection from CTL-mediated cytolysis, has been demonstrated in several systems including in the maintenance of the "immune privilege" of the eye (Griffith et al., 1995), the testis (Bellgrau et al., 1995) and also in melanoma- (Hahne et al., 1996) or colon cancer-mediated defense against reactive CTLs (O'Connell et al., 1996).

The numbers of activated CTLs present in the lamina propria and also in the intraepithelial cell compartment in the intestinal mucosa of patients with active IBD are quite impressive - but what are the consequences of such an enhanced cell-mediated cytotoxicity reaction in the mucosa? Indications for the possible significance of local cell-mediated cytotoxicity on the pathogenesis of IBD come from recent studies on the production of cytokines and chemokines by intestinal epithelial cells. When expression of the C-X-C chemokine ENA-78 ("neutrophil activating peptide-78") (Walz et al., 1991) was assessed by in situ hybridization and by immunofluorescent staining in patients with active IBD and normal controls, it became evident that this potent neutrophil-recruiting and activating chemokine is preferentially expressed at the mRNA level on tissue specimens with early signs of disease activity. ENA-78 immunoreactive protein has been detected in the overwhelming majority of epithelial cells at these early stages of disease activity (Z'Graggen et al., 1997). When the localization of ENA-78 protein positive epithelial cells is compared with the localization of

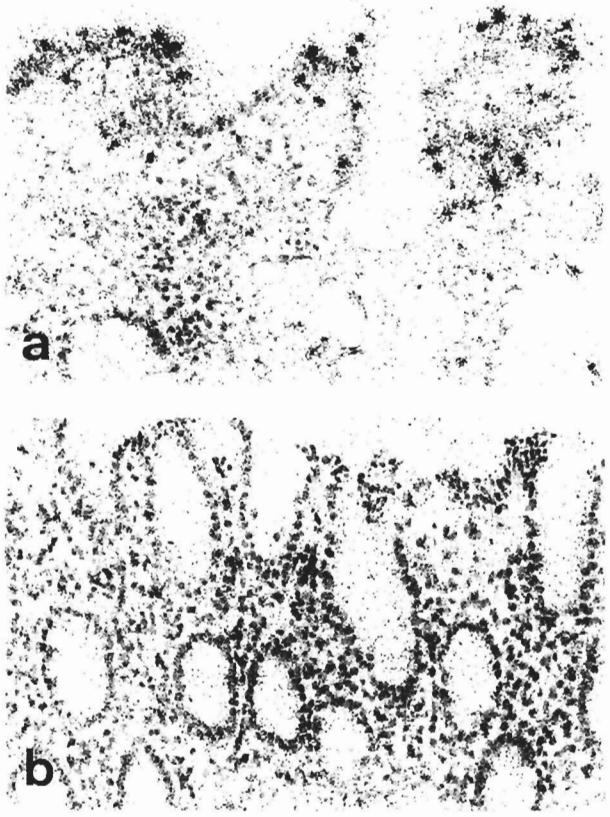

Fig. 4. Detection of perforin mRNA expressing cells by in situ hybridization in colonic tissues from a patient with active ulcerative colitis **(a)** , and a normal control **(b)**. x 150

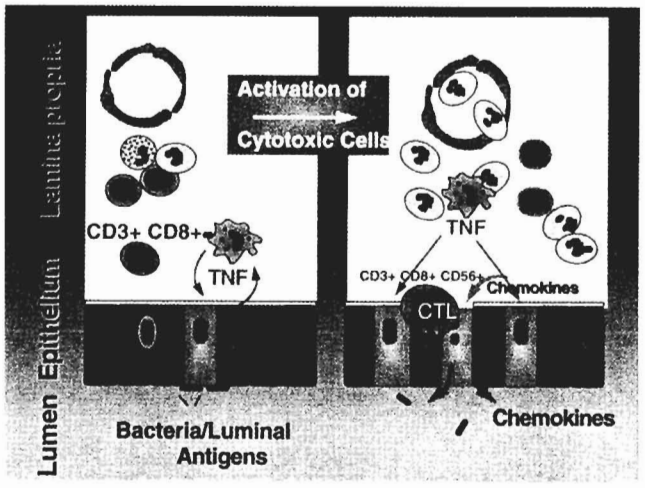

Fig. 5. Hypothetical early alterations in the intestinal mucosa of patients with inflammatory bowel disease: luminal antigen vs induce the production of pro-inflammatory cytokines and chemokines by epithelial cells and macrophages of the lamina propria. Cytotoxic T cell-mediated cytolysis of epithelial cells leads to the increased release of chemokines and cytokines into the lamina propria resulting in an increased permeability of the epithelium and an increased recruitment of inflammatory cells to the intestinal mucosa.

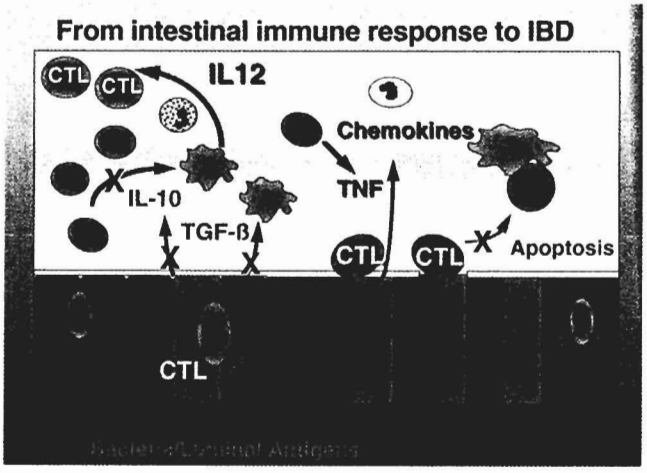

Fig. 6. Factors that are potentially involved in the transition from a physiologic, tightly controlled immune reaction to the excessive immunopathological reaction observed in the intestinal mucosa from patients with IBD.

activated, perforin mRNA expressing cells, it becomes obvious that a considerable fraction of activated CTLs are close to, or even in direct contact with, ENA-78-containing cells. Hence, CTL-mediated lysis of intestinal epithelial cells may lead to the release of cytokines and chemokines produced and - at least in part - stored by these epithelial cells. This CTL-mediated release of chemokines and cytokines may in turn lead to the increased recruitment and activation of inflammatory cells, thus further amplifying the inflammatory reaction. Schematically these steps are depicted in Fig. 6. Based on recent work of several groups using established intestinal epithelial cells it became evident that ENA-78 is not the only cytokine produced by intestinal epithelial cells. The pro-inflammatory cytokine TNF-α, IL-1ß, but also further chemokines such as IL-8 or MCP-1 are produced by epithelial cells upon cellular activation, such as infection by invasive bacteria or binding of bacteria to the luminal side of epithelial cells (Yang et al., 1997). The presence of activated perforin mRNA expressing CTLs in the intestinal mucosa is not unique to active IBD. In fact, occasionally even in tissue samples from normal controls, elevated frequencies of activated CTLs are found in limited mucosal areas and also the expression of ENA-78 in intestinal epithelial cells is not unique to IBD since in acute appendicitis comparable strong ENA-78 mRNA expression in the epithelial cells at the site of inflammation is observed (Z'Graggen et al., 1997; Müller et al., 1998). It is thus the combination of increased frequencies of activated CTLs in extended areas of the intestinal mucosa together with the enhanced expression of pro-inflammatory chemokines that contributes to the deleterious inflammatory tissue destruction observed in IBD.

Factors that may contribute to an exaggerated cell-mediated immune response in IBD

The exact reasons for the observed hyperreactivity of the cell-mediated cytotoxicity in active IBD have not been defined yet. Several hypothetical pathways, that are not mutually exclusive, can be envisaged to explain for the observed strong activation of CTLs (Fig. 5): (i) the persistence of antigens in the intestinal mucosa leads to the continued production of pro-inflammatory cytokines, such as TNF-α or IL-1 and chemokines, leading to an enhanced recruitment and activation of inflammatory cells; (ii) recruited and resident CD4 T cells differentiate preferentially into Th1 cells, thus favoring the generation of a potent cell-mediated cytotoxic immune reaction; and (iii) the inactivation or elimination of activated T cells in the intestinal mucosa due to activation induced cell death is impaired, hence, activated T cells are operative for an extended time, leading to a deleterious accumulation of potent effector cells.

An enhanced persistence of antigens in the intestinal bowel wall can be caused by an increased permeability of the intestinal epithelium and of the mucus layer on the luminal side, and of the basal membrane at the baso-lateral side of the intestinal epithelium. Experimental evidence for altered permeability of the intestinal epithelium causing an CD-like disease has been provided in a mouse model (Hermiston and Gordon 1995). Furthermore, persisting bacterial or virus infections of the intestinal mucosa, e.g. with *M. paratuberculosis* (Mishina et al., 1996), measles virus (Ekbom et al., 1996) have also been considered relevant observations for the etiology of IBD. Persistence of antigen may lead to continued secretion of pro-inflammatory cytokines. Among these factors, TNF-α seems to represent a key mediator as convincingly demonstrated by the beneficial effects of an anti-TNF treatment in patients with CD (Targan et al., 1997). The TNF-mediated effects crucial for the pathogenesis of IBD are unknown so far. Among the pleiotropic effects mediated by TNF, up-regulation of MHC- and adhesion molecule-expression, induction of genes encoding other pro-inflammatory cytokines including various chemokines, but also more direct effects, such as induction of accelerated apoptosis of intestinal epithelial cells with subsequent shedding into the gut lumen (Guy-Grand et al., 1998), thus affecting the permeability of the intestinal epithelium, have been postulated to represent major pathogenetically relevant effects. The requirement of a tightly controlled mucosal immune system in the intestine for the maintenance of the local tissue homeostasis has been convincingly demonstrated in mice deficient for genes involved in the maintenance of a balanced immune response. Hence, the absence of critical immune regulatory cell subsets such as the recently characterized IL-10 and TGF-ß producing Tr1 cells (Groux et al., 1997) may lead to the generation of an exaggerated cellular immune response. The observed increased local production of IL-12 in the affected mucosa of patients with IBD further confirms the present notion that the preferential differentiation of resident and recruited T cells into cytotoxic effector cells may represent a key step in the pathogenesis of IBD. IL-12 favors a preferential differentiation of CD4 T cells into Th1 cells, and further enhances the cytolytic potential of cytotoxic effector cells. An accumulation of activated CTLs observed in active IBD may not only be due to increased recruitment and activation of effector cells, but also due to an impaired elimination of activated effector cells. Hence, studies dealing with the mechanisms operative in the elimination of activated T cells ("activation-induced cell death") in normal intestinal mucosa, and in the affected mucosa of patients with active IBD are currently the focus of intense research activities.

In conclusion, increasing evidence suggests that immunopathological mechanisms, in particular cell-mediated cytotoxic reactions, play a central role in the initiation and perpetuation of inflammatory bowel disease. Although quantitative differences in the extent of activation of CTLs exist between CD and UC, with higher frequencies of activated mucosal CTLs observed in patients with active CD, differences in the

histopathological alterations mediated by the cellular immune system in these two entities are minimal. This supports the currently favored hypothesis that IBD represents a number of different conditions that eventually result in the same tissue response with similar histopathological alterations at advanced stages of disease. This concept is further supported by the similarity of the clinical and histopathological appearance of colitis in different mouse models including mice deficient for distinct genes.

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Invited Review

The visualization of oxidant stress in tissues and isolated cells

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Summary. Many studies have implicated the role of oxidant stress in a wide range of human diseases and have led to the rapid expansion of research in this area. With many experimental approaches a direct detection of the production of reactive oxygen species (ROS) and free radicals is not possible. Free radicals are very reactive, short-lived and react in a non-specific way, so that ongoing oxidative damage is generally analyzed by measurement of secondary products e.g. H_2O_2, "oxidized" proteins, peroxidized lipids and their break-down products, "oxidized" DNA or by fluorographic analysis in combination with fluorescent dyes e.g. dichlorofluorescin (DCFH). The histochemical visualization of selected molecular markers for oxidative phenomena can often provide valuable information concerning the distribution of oxidative processes *in vivo*. A number of biochemical methods are available for the monitoring of almost all oxidant stress-related processes, although their applicability *in vivo* is limited.

This review summarizes the biochemical methods currently available for histochemical detection and indirect visualization of an excess of free radicals and ROS. The cited methods are discussed and the results obtained from their application are critically evaluated.

Key words: Oxidant stress, Histochemical detection, Marker, ROS

Introduction

The comprehensive term "oxidant stress" is currently employed to indicate a complex series of biochemical processes, which can affect living matter under several distinct conditions and produce both physiological and pathological effects. Oxidant stress can be loosely defined as the prevalence within the living cell of oxidizing species over the cellular antioxidant defences, and it is this 'pathological' perspective that has received the most attention over the past decades. Until recently, a considerable amount of research has in fact focused on the role of oxidant stress in mediating the cell damaging effects of a wide range of prooxidant agents (chemicals, drugs, pollutants); several detailed reviews have been published, dealing with the mechanistic aspects of these cytotoxic processes (Comporti, 1989; Farber et al., 1990; Janssen et al., 1993; Rubbo et al., 1996; Wiseman and Halliwell, 1996; Pompella, 1997).

At the same time, a number of studies in recent years have highlighted the role of oxidation/reduction (redox) reactions in the regulation of molecular mechanisms involved in cellular signal transduction (Powis et al., 1995; Lander, 1997; Nakamura et al., 1997; Sen, 1998). It is becoming clear that low "physiological" levels of oxidant agents (and reactive oxygen species in particular) can have physiological roles within cells; thus, the term "oxidant-mediated regulation" has recently been proposed as a more accurate alternative to "oxidant stress" (Cotgreave and Gerdes, 1998). This novel perspective is particularly intriguing, as it will surely advance our understanding of the role played by redox processes in human disease; oxidant stress has in fact been recognized as a primary factor in the pathogenesis of a number of notable human pathologies, such as atherosclerosis, ischemia-reperfusion, cancer or Alzheimer's disease (reviewed by Halliwell and Gutteridge, 1989).

Sophisticated and sensitive biochemical procedures are available for the determination of even minimal levels of oxidant stress *in vivo* (Packer, 1994). However, such approaches do not generally allow collection of information concerning the distribution of such phenomena *in situ*, even though this aspect is of great potential importance for the understanding of oxidative processes especially in the case of tissues with a heterogeneous cell composition, such as brain, lung or kidney. As a result, some laboratories - including our own - have approached the issue of determination of oxidant stress *in vivo* from a histochemical point of view. The possibility of developing specific and sensitive procedures able to directly reveal some of the biochemical changes induced by oxidant stress have

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therefore made feasible the discrimination of areas, cellular types and – possibly – subcellular sites which are involved in this phenomenon. This review will present an updated survey of the available histochemical approaches, of the problems encountered with some of them, and of the most relevant applications published in the literature.

Visualization of sites of production of reactive oxygen species (ROS)

ROS (superoxide anion, hydrogen peroxide, hydroxyl radical, singlet oxygen) are continuously produced during normal cellular metabolism at several different sites, e.g. mitochondrial respiratory chain, cytochromes P450 and b5, xanthine oxidase, and the NADPH-oxidase system of phagocytic cells (Halliwell and Gutteridge, 1989). Under several pathophysiological conditions, this 'basal' production of ROS can be considerably increased (inflammatory disease, ischemia-reperfusion). ROS are also produced by the action of a number of xenobiotics (Comporti, 1989) and 'redox-cycling compounds' (Kappus and Sies, 1981), including several drugs and transition metal ions, in particular iron. Figure 1 presents a simplified outline of the main sources of ROS and of their interconversions and interactions with radical nitrogen species.

The specific detection of superoxide anions in biological samples is made difficult by several methodological problems (Fridovich, 1997) and therefore requires the most accurate application of specificity controls. For many years, especially in studies using activated phagocytes, the reduction of nitroblue tetrazolium (NBT) to insoluble blue formazan has been widely used to evaluate superoxide production at light microscope level, as well as with electron microscopy (Hirai et al., 1992). However, the cytochemical visualization of subcellular sites of ROS production has predominantly been made possible by the outstanding contributions of M.J. Karnowsky and his coworkers, who defined and optimized the procedures for detection of hydrogen peroxide (by the cerium chloride method) and superoxide (by the Mn^{2+}-diaminobenzidine method), additionally providing insights into the cellular production of singlet oxygen (reviewed by Karnowsky, 1994). Using activated neutrophils, the cerium chloride method has also recently been used for detection of cellular sites of H_2O_2 production with the laser-scanning confocal reflectance microscope (Robinson and Batten, 1990). In addition to experiments with phagocytes, the same methods have successfully been employed for the demonstration of the

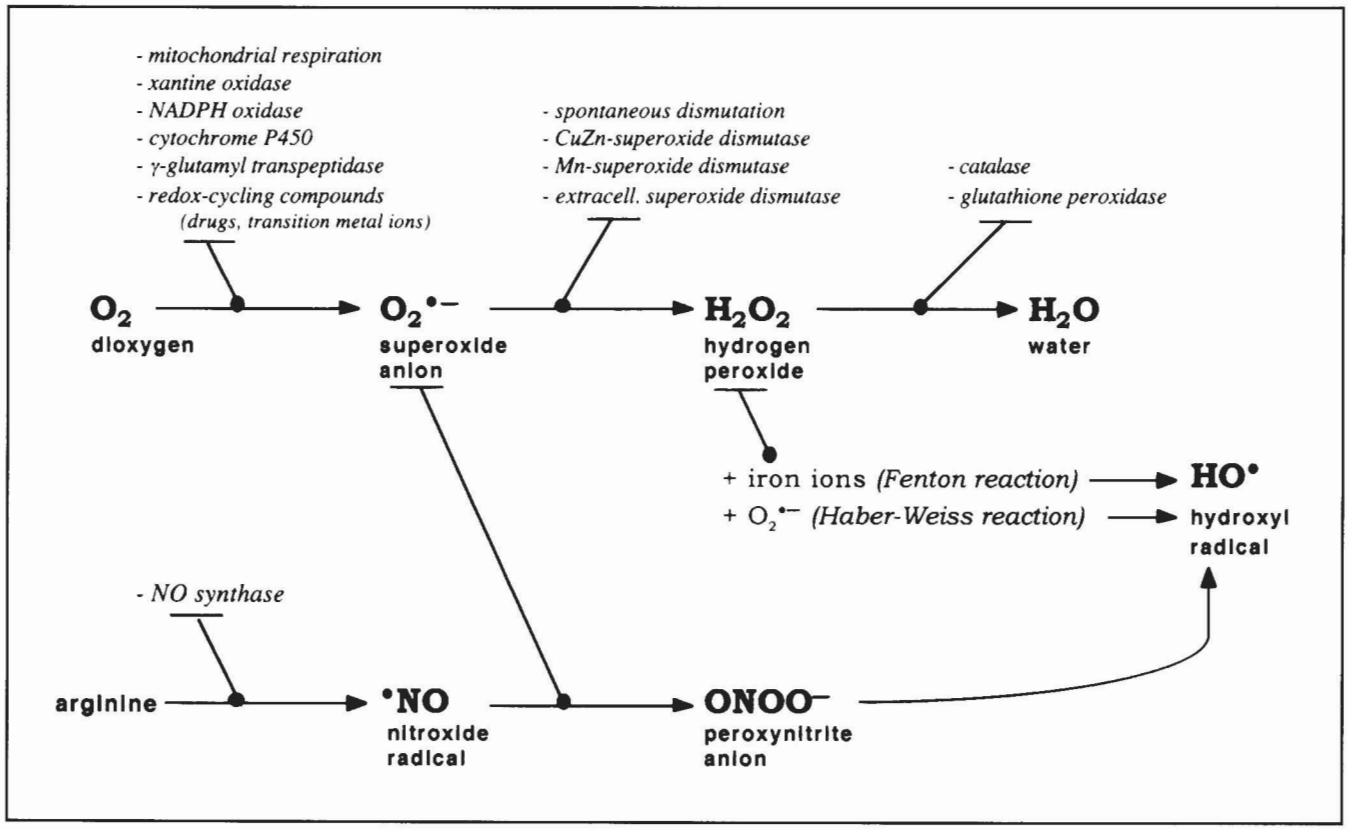

Fig. 1. Main processes involved in formation of reactive oxygen species, their interconversions and their interactions with nitrogen-centered reactive species.

production of ROS at the endothelial surface of cardiac vessels, during the first moments of reperfusion following a period of anoxia (Schlafer et al., 1990; Babbs et al., 1991).

Studies aimed at revealing the production of ROS are also currently being carried out using a method involving the preloading of living cells with 2',7'-dichlorofluorescin diacetate (DCF-DA), a compound whose fluorescence sharply increases in the presence of superoxide and other oxygen radical species (Fig. 2). DCF-DA has thus been successfully employed in the investigation of whole organs and isolated cells, as well as in flow cytometry applications (Royall and Ischiropoulos, 1993; Tsuchiya et al., 1994). Even so, the actual specificity of DCF-DA in detecting individual ROS has never been elucidated with sufficient detail; one interesting investigation in this direction has indeed questioned the ability of DCF-DA to detect superoxide anion, and has shown that DCF-DA can itself serve as a substrate for xanthine oxidase and other cellular peroxidases (Zhu et al., 1994).

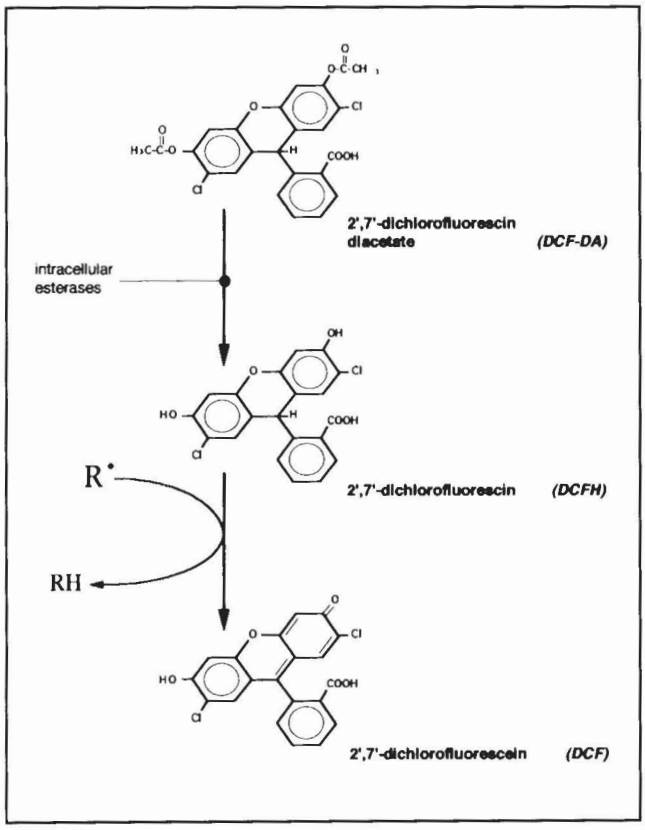

Fig. 2. Detection of oxidants within living cells by means of 2',7'-dichlorofluorescin diacetate (DCF-DA). Once DCF-DA has penetrated into the cell, cytosolic esterases will promptly release the non-cell permeable compound, DCFH, which is thus trapped in the cell. The observation of the oxidation of DCFH to the highly fluorescent derivative DCF is interpreted as the visible effect of oxidant conditions taking place intracellularly.

Visualization of aldehydes and carbonyls derived from the peroxidation of unsaturated lipids

Under selected circumstances, the progression of oxidant stress within a cell can lead to the initiation of a highly destructive, self-propagated chain of oxidative reactions involving cell membrane lipids, a process termed lipid peroxidation (LPO). This process ultimately results in the oxidative fragmentation of polyunsaturated fatty acids of cellular phospholipids, and includes a complex series of biochemical steps which have been described in detail by several authors (Horton and Fairhurst, 1987; Halliwell, 1990; Esterbauer et al., 1991). With respect to histochemistry, the most prominent aspect of LPO is the formation of a wide range of aldehyde and carbonyl compounds following the breakdown of polyunsaturated fatty acids; a comprehensive description of such products has been provided by Esterbauer et al. (1989). Of these LPO-derived products, the lipophilic ones will remain associated with the lipid phase, while others (e.g. malonaldehyde, MDA, one of the most abundant LPO products) will promptly diffuse into aqueous media. An important class of LPO products is the α,ß-unsaturated aldehydes, which show variable degrees of reactivity with amino acid residues in protein. Altogether - as shown schematically in Figure 3 - the process of LPO will result in a marked increase in the amount of carbonyl and aldehyde groups in cellular lipids and proteins, along with a decrease in protein reduced thiol groups. Thus, the occurrence of LPO in a given specimen can be inferred by the histochemical identification of these biochemical alterations.

The direct Schiff reaction has long been employed for the identification of aldehydes in tissues, even though the exact mechanism for the generation of chromogen has never been convincingly elucidated, despite a long-lasting debate (see e.g. Hörmann et al., 1958). With respect to LPO-derived aldehydes, Schiff's reaction was first used for the visualization of areas with decreased sensitivity to induction of lipid peroxidation *in vitro* in cryostat sections obtained from the liver of rats following administration of a carcinogen (Benedetti et al., 1984). Subsequently, the same procedure was applied to the detection of lipid peroxidation in vivo in the whole animal (Pompella et al., 1987), using a model involving intoxication with bromobenzene, a glutathione-depleting agent with a strong prooxidant action on several rat organs (Comporti, 1987). Figure 4 shows the distribution of LPO in mouse liver, as assessed by the direct Schiff reaction, after treatment with bromobenzene and with allyl alcohol, another distinct glutathione-depleting prooxidant.

The direct Schiff reaction was subsequently employed with success under other experimental conditions, allowing the demonstration of the selective involvement of rat *substantia nigra* during *in vitro* iron-induced lipid peroxidation (Tanaka et al., 1992), and of rat tubular proximal epithelium during *in vivo* lipid

peroxidation induced by the nephrocarcinogen iron nitrilotriacetate (Toyokuni et al., 1990). Very interesting results were obtained by Masuda and Yamamori, who studied the distribution of LPO in relation to that of cell injury in rat livers subjected to anterograde *vs.* retrograde perfusion with different prooxidant toxins and under different oxygen tensions (Masuda and Yamamori, 1991a,b).

However, the use of Schiff reaction is limited by a somewhat poor reproducibility, and the strong acidity of the reagent can induce false positivite results in tissues rich in plasmalogens, such as myocardium, where the so-called "pseudoplasmal" reaction can be seen (reviewed in Pearse, 1985). These difficulties have therefore warranted the development of alternative procedures. Good results have been obtained using a reaction based on 3-hydroxy-2-naphthoic acid hydrazide (NAH) followed by coupling with a tetrazolium salt (Fig. 5); the reliability of the NAH reaction has been assessed by means of microspectrophotometrical analysis of tissue sections and comparison with data obtained by biochemical determination of LPO in the same specimens (Pompella and Comporti, 1991). The use of the NAH reaction allowed visualization of regions first affected by lipid peroxidation *in vivo* following the intoxication with haloalkanes (carbon tetrachloride, bromotrichloromethane); such LPO levels had proven to be lower than the detection limit possible with the direct Schiff reaction.

A further improvement in the histochemistry of lipid peroxidation was obtained by the employment of fluorescent reagents for the identification of LPO-derived carbonyls in tissues and isolated cells. Fluorochromes have in fact enabled an appreciable increase in the sensitivity of detection together with the possibility of analysis by means of confocal laser scanning fluorescence microscopy with image video-analysis. Interesting results with this procedure have been obtained by exploiting the fluorescence of the NAH reagent itself (Pompella and Comporti, 1993). An example of results obtainable with this procedure is shown in Figure 6, where the selective involvement of the first rows of pericentral hepatocytes by lipid peroxidation induced *in vivo* in the rat by intoxication with carbon tetrachloride can be seen. An alternative approach to fluorescent derivativization of cellular

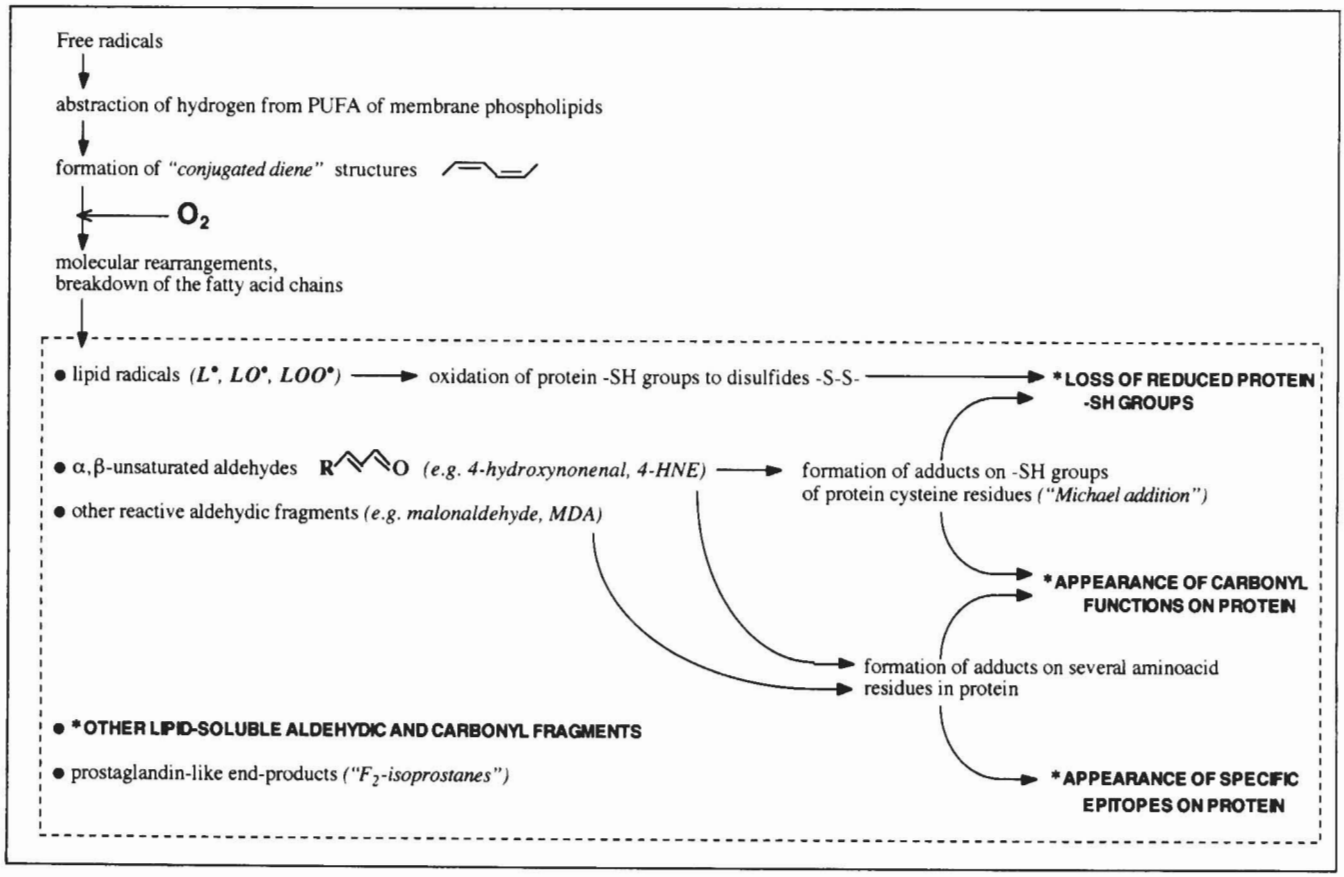

Fig. 3. Main products of lipid peroxidation and their modifying effects on proteins. Several classes of compounds are originated during the process of lipid peroxidation, some of which are directly detectable by histochemical procedures and/or lead to histochemically-detectable alterations in proteins (indicated with an asterisk).

carbonyls together with confocal laser scanning microscopy was followed by others, using a biotin-labelled hydrazide coupled with fluorescent-conjugated streptavidin (Harris et al., 1994).

An additional tool for the detection of LPO is the naturally fluorescent fatty acid, *cis*-parinaric acid. Once pre-loaded in living cells, *cis*-parinaric acid is readily consumed during lipid peroxidation, thus allowing the monitoring of the lipid peroxidation process in the form of a fluorescence decrease. To date, the procedure has found successful application in flow cytometry (Hedley and Chow, 1992).

Visualization of oxidative changes in cellular protein ('protein oxidation')

The oxidative modification of proteins by reactive oxygen species and other reactive compounds has been recognized to play a role in the progression of several pathophysiological processes, including a range of notable diseases and aging (Davies and Dean, 1997).

As comprehensively reviewed by Stadtman and Barlett (1997), there are three major pathways leading to protein oxidation: i) the so-called metal-catalyzed oxidation of amino acid residues; ii) lipid peroxidation; and iii) protein glycation and glycoxidation reactions (Fig. 7). In the case of metal-catalyzed protein oxidation, the direct oxidation of amino acid residues is the consequence of interaction with the polypeptide chain of hydroxyl radicals, generated e.g. by ionizing radiation, or by the Fenton reaction between H_2O_2 and iron ions (Fig. 1). Almost all amino acid residues can be involved, and the process often results in peptide bond cleavage and protein-protein cross link formation.

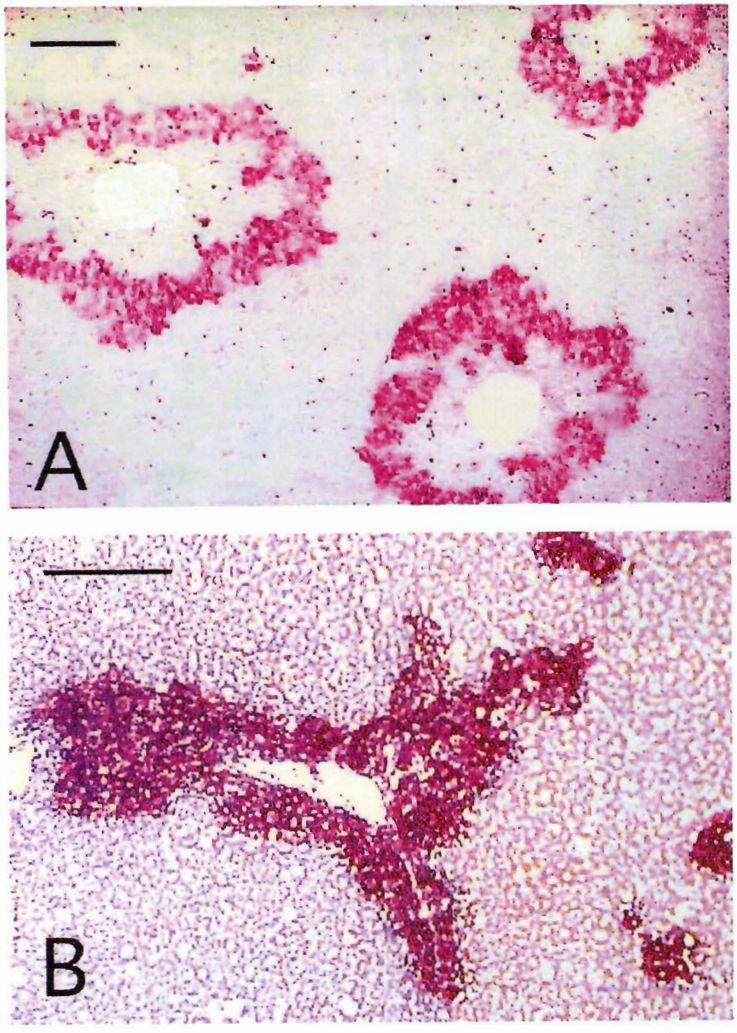

Fig. 4. Visualization by direct Schiff reaction of tissue areas becoming involved by lipid peroxidation *in vivo* in mouse liver, following the administration of glutathione-depleting toxins. **A.** Medio-lobular distribution of LPO (purple stain) caused by bromobenzene intoxication. **B.** Strict periportal localization of LPO induced by intoxication with allyl alcohol. See Pompella et al. (1987) for details of the procedures employed. Bars: 50 μm.

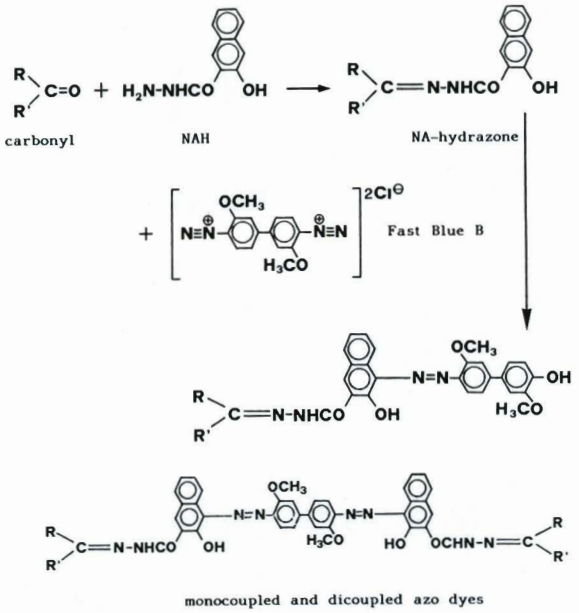

Fig. 5. The naphthoic acid hydrazide-fast blue B (NAH-FBB) reaction or detection of carbonyl groups in tissues (Pompella and Comporti, 1991). Carbonyls are first converted to naphthoic hydrazones by reaction with NAH; the coupling with the diazonium salt yields then violet-coloured azo dyes.

A second pathway leading to protein oxidation is that of lipid peroxidation. As discussed in the previous section, the involvement of proteins in reactions with LPO-derived reactive compounds can in fact result in an increase in protein-associated carbonyl functions, along with a decrease in detectable reduced protein thiols (Fig. 3). This is the result of a direct addition of α,ß-unsaturated aldehydes to amino acid residues in protein, which can lead to the formation of adducts in which the aldehyde grouping remains available for further reactions (Fig. 8). Malonaldehyde, which is one of the most abundant LPO aldehydic products, has also been shown to introduce carbonyl functions in protein (Burcham and Kuhan, 1996).

Glycation (i.e. the binding of glucose to protein) is commonly observed in diabetes mellitus patients, and can facilitate protein oxidation. Combined glycation and oxidation, 'glycoxidation', occurs when oxidative reactions affect the initial products of glycation, and results in irreversible structural alterations in proteins; this eventually leads to the accumulation of the so-called 'advanced glycation end-products' (AGEs) in tissue (Vlassara et al., 1994).

For the detection of protein-associated carbonyl functions, the method originally developed by Levine et al. (1994) has recently been adapted in our laboratory for histochemical application (Pompella et al., 1996a,b). As demonstrated in Figure 9, the procedure consists of a first step, in which protein carbonyls are derivativized by 2,4-dinitrophenyl hydrazine (2,4-DNPH) to yield the corresponding 2,4-dinitrophenyl hydrazones. In a second step, the dinitrophenyl (DNP) groups which become associated with proteins in this way are detected immunochemically by means of a commercial anti-DNP antiserum; finally, antibodies bound to specimens are identified with a conventional biotin-avidin system, or equivalent (Pompella et al., 1996a).

In principle, the 2,4-DNPH/anti-DNP procedure should reveal all kinds of carbonyls becoming associated with protein, irrespective of their origin. With this method, oxidized proteins have been visualized in several interesting studies, e.g. in activated neutrophil

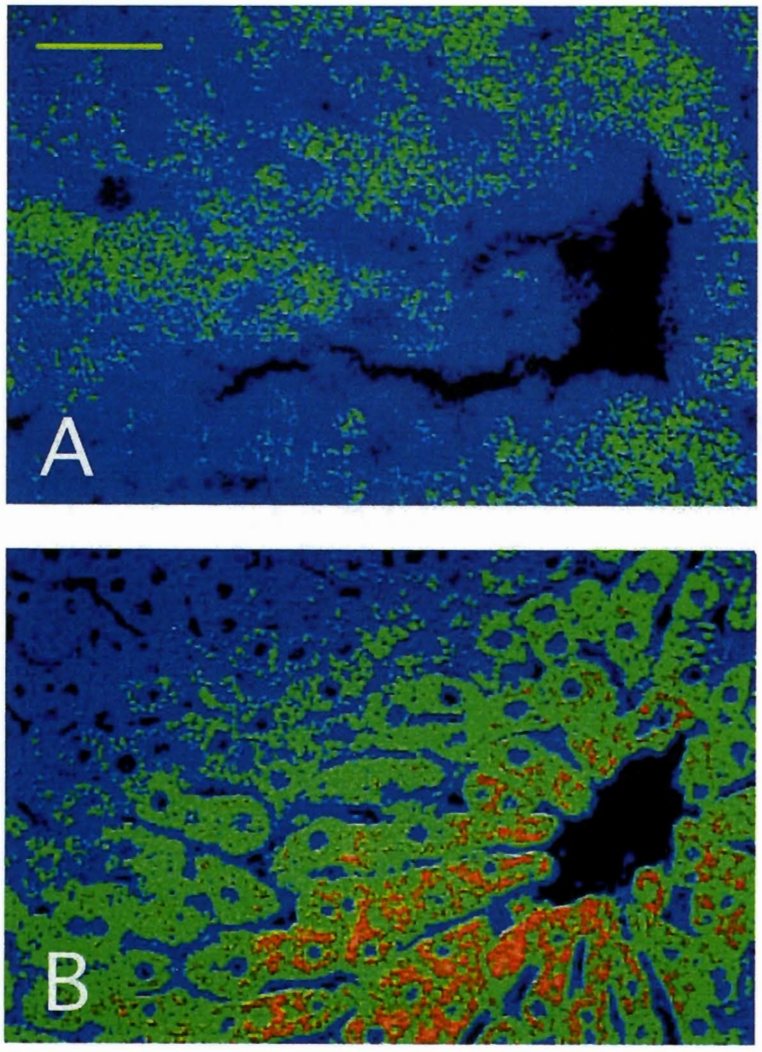

Fig. 6. Confocal laser scanning fluorescence imaging of lipid peroxidation in rat liver in vivo, following fluorescent derivativization of cellular carbonyls with NAH. Fluorescence intensities are translated into false-color images generated by the computer, according to a scale going from blue (lower intensities) to green, yellow (middle intensities) and red (higher intensities). **A.** Control rat liver. **B.** Rat liver following intoxication with the prooxidant haloalkane carbon tetrachloride. See Pompella and Comporti (1993) for details of the procedures employed. Bar: 50 μm.

phagocytes (Pompella et al., 1996a; Cambiaggi et al., 1997), in brain tissue from Alzheimer patients (Smith et al., 1996) and in sarcoma cells exposed to prooxidant treatments (Fig. 10) (Frank et al., 1998). However, the usefulness of the 2,4-DNPH/anti-DNP procedure for the histochemical detection of protein glycoxidation products has not yet been evaluated.

Another important parameter which can be used as a marker of oxidant insult to protein is the loss of reduced -SH groups, which has mainly been investigated with reference to toxic cell injury and lipid peroxidation (Bellomo and Orrenius, 1985; Pompella et al., 1991). Modern histochemistry of protein thiols was developed and optimized by G. Nöhammer, who laid down the foundations for semi-quantitative microspectrophotometry of protein reduced thiols, protein disulfides and protein mixed disulfides (Nöhammer, 1981, 1982). These procedures have found interesting applications in the histochemical evaluation of protein thiol redox status in neoplastic and preneoplastic cells (Nöhammer et al., 1986; Pompella et al., 1996b). Fluorescent labelling procedures have recently been developed for the visualization of both total and cell surface protein reduced thiols at the single cell level, by laser-scanning confocal microscopy (Pompella et al., 1996a) (Fig. 11).

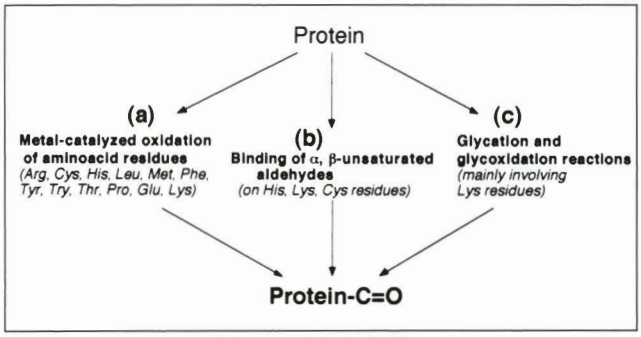

Fig. 7. Three distinct pathways to protein oxidation (= increase of carbonyl groups in proteins). Carbonyl groups are introduced into proteins **(a)** as a result of direct oxidant attack to protein, through the metal-catalyzed oxidation of side chains of several amino acids, **(b)** following a process of lipid peroxidation, by the reaction of the double bond of alpha, beta-unsaturated aldehydes (e.g., 4-HNE) with amino, sulfhydryl and imidazole groups in protein, and **(c)** by reaction of protein amino groups with carbohydrates, through glycation and glycoxidation reactions.

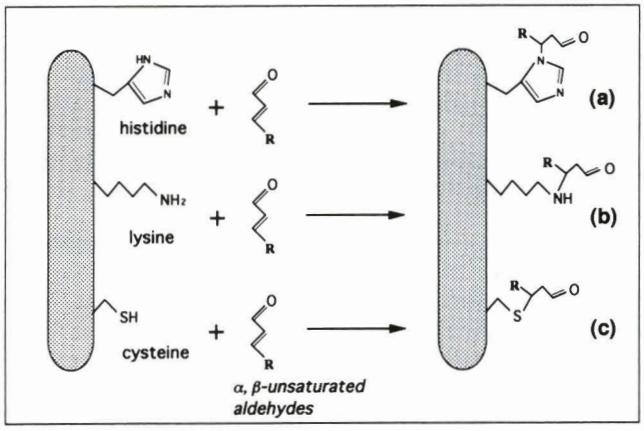

Fig. 8. Protein carbonyl adducts resulting from the reaction of selected amino acid side chains with LPO-derived alpha, beta-unsaturated aldehydes. The imidazole moiety of histidine residues, the epsilon-amino group of lysine residues, and the sulfhydryl group of cysteine residues, may all undergo addition reactions with the alpha, beta-double bond of alpha, beta-unsaturated aldehydes (e.g., 4-HNE), to form **(a)** the corresponding tertiary amine derivatives, **(b)** the secondary amine, and **(c)** thioester structures.

Immunodetection of oxidant stress-induced epitopes in proteins and nucleic acids

The immuno-histochemical approach to oxidant stress has expanded rapidly over the past few years, and today represents the tool of choice for the specific detection of oxidative changes in tissue and cells. Following the structural alterations introduced by an oxidant insult, proteins can in fact easily acquire new antigenic properties due to the appearance of new specific epitopes on the polypeptide chain. This is primarily the case with reactive aldehydes derived from lipid peroxidation, which are able to bind to several amino acid residues, as outlined in Figure 3. By means of specific polyclonal or monoclonal antibodies, the occurrence of malonaldehyde (MDA) and 4-hydroxy-nonenal (4-HNE) bound to cellular protein has thus been

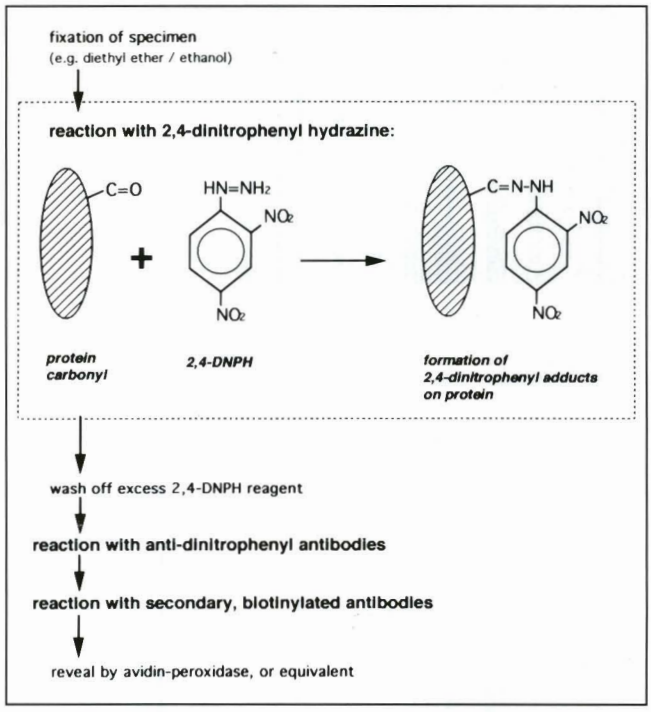

Fig. 9. The two-step procedure for the histochemical determination of protein carbonyl groups. See Pompella et al. (1996a) for details.

documented under a number of experimental and clinical conditions. Lipid peroxidation has been demonstrated in this way in collagen-producing fibroblasts (Chojkier et al., 1989; Bedossa et al., 1994), in the liver of human alcoholics (Niemelä et al., 1994), hepatitis C patients and other chronic liver diseases (Paradis et al., 1997a,b), in the arterial wall during experimentally-induced atherosclerosis (Palinski et al., 1989), in activated neutrophils (Quinn et al., 1995; Cambiaggi et al., 1997), in nigral neurons of Parkinson patients (Yoritaka et al., 1996), in ferric-nitrilotriacetate-induced renal carcinogenesis (Uchida et al., 1995) as well as in human renal carcinoma (Okamoto et al., 1994). Most of these studies were largely made possible by the availability of thoroughly characterized monoclonal antibodies (Toyokuni et al., 1995; Waeg et al., 1996).

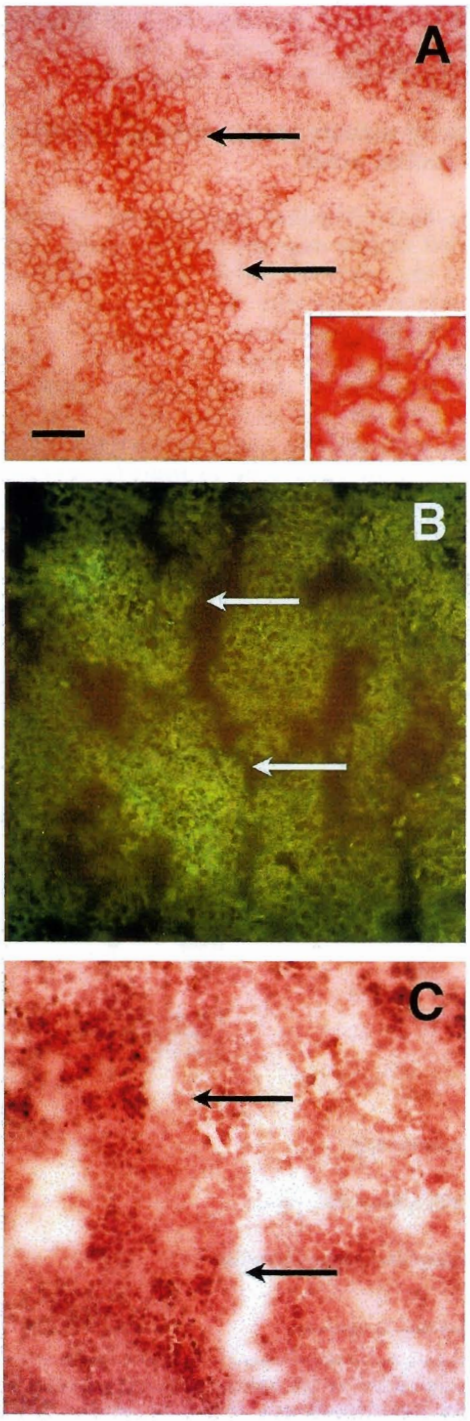

Fig. 10. Colocalization of protein oxidation (**A**, DNPH/anti-DNP reaction) and lipid peroxidation. (**B**, immuno-fluorescence by polyclonal antiserum against protein-4-HNE adducts) at sites of cellular injury, (**C**, hematoxylin/eosin). Rat DS-sarcoma cells treated in vivo with hyperthermia plus respiratory hyperoxia and xanthine oxidase. Bar: 50 μm. Reproduced from Frank et al. (1998).

Specific epitopes are also present in oxidized low-density lipoproteins (ox-LDL), a distinctive class of oxidized proteins probably involved in the pathogenesis of atherosclerosis. The exact nature of such epitopes is a matter of debate, although is seems certain that the antigenicity of oxLDL can be at least partially accounted for by the binding of LPO-derived aldehydes, such as MDA and 4-HNE, to the LDL apoprotein moiety (Chen et al., 1992; O'Brien et al., 1996; Requena et al., 1997). By means of polyclonal and monoclonal antibodies raised against in vitro-oxidized LDL, the immunohistochemical visualization of ox-LDL has been repeatedly reported in atherosclerotic lesions (Palinski et al., 1989; Ylä-Herttuala et al., 1990; Paolicchi et al., 1999).

With respect to AGE-modified proteins, immunohistochemical studies aimed at determining the sites of accumulation of these products have generally employed antibodies specific for N-ε-(carboxymethyl) lysine, the main antigenic structure produced during protein glycoxidation (Ikeda et al., 1998). In this way, AGEs have recently been detected in several disease conditions (Matsuse et al., 1998; Sasaki et al., 1998; Sun et al., 1998; Takayama et al., 1998).

Besides protein oxidation, resulting in increased levels of protein carbonyls, a different type of oxidative attack on protein must also be considered, i.e. that caused by reactive nitrogen species (Fig. 12). The peroxynitrite anion $ONOO^-$ can in fact react with aromatic amino acids, probably through the formation of the nitronium cation NO_2^+ and of the $^{\bullet}NO_2$ radical. This will primarily result in the addition of nitrate groups to the ortho position of tyrosines, a process referred to as 'protein nitration' (Darley-Usmar et al., 1995). Histochemically, protein nitration can be documented by means of immunomethods employing anti-nitrotyrosine antibodies (Beckman et al., 1994; Good et al., 1996; Virág et al., 1998).

Finally, recent studies have explored the possibility of extending histochemical investigations to the detection of oxidized DNA. DNA damage by both reactive oxygen and reactive nitrogen species is in fact a prominent aspect of a number of oxidant stress conditions. Toyokuni and coworkers have developed a quantitative immunohistochemical procedure for determination of 8-hydroxy-2'-deoxyguanosine - one of the main new epitopes on oxidized DNA - by means of a specific monoclonal antibody (Toyokuni et al., 1997).

Final remarks

Through the development of numerous methods and

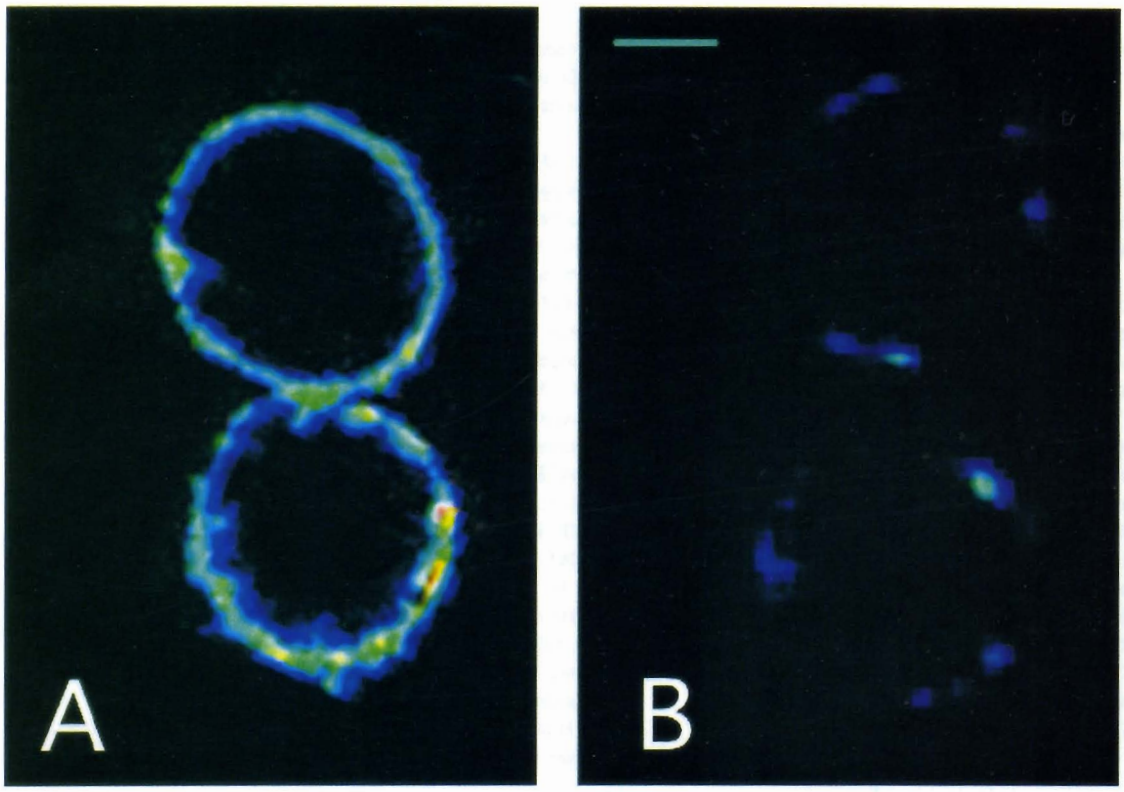

Fig. 11. Selective visualization of cell surface reduced protein sulfhydryl groups by MPB-based immunofluorescence. Confocal laser scanning fluorescence imaging; see Pompella et al. (1996a) for details of the procedures employed. **A.** Control HPBALL leukemia cells, showing a ring of reduced protein sulfhydryls at their surface. **B.** Loss of surface protein sulfhydryls following the exposure of cells to the lipid peroxidation product, 4-hydroxynonenal (4-HNE). Bar: 10 μm.

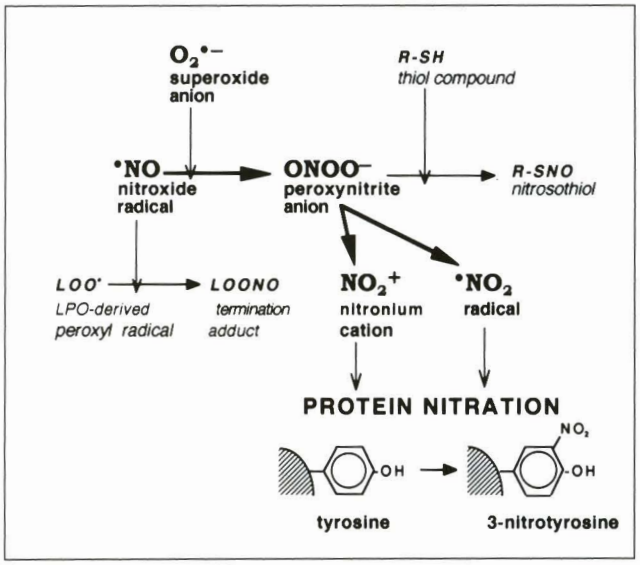

Fig. 12. Reactions of the reactive nitrogen species, nitroxide radical and peroxynitrite anion, leading to the formation of adducts on protein tyrosine residues ("protein nitration").

specific agents as outlined in this review, histochemistry has now become one of the most important experimental approaches available to researchers investigating oxidant stress. Although oxidative alterations have long been known to be associated with several disease conditions, the actual involvement of oxidant stress in the *pathogenesis* of a given condition has been convincingly documented only in a small number of cases to date. When compared to even the most sophisticated and specific biochemical determinations, the histochemical approach to evaluating oxidant stress offers the advantage of providing a 'certification' that this process is occurring at a given time and at a given site, either *in vitro* or *in vivo*. Information obtained in this way can often add significant details to an investigation of oxidative alterations in biological samples, thus assisting in the elucidation of the precise role of oxidant stress in experimental and clinical processes.

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