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REVIEW ARTICLE

THE B LYMPHOCYTE: A NEWLY RECOGNIZED SOURCE OF REACTIVE OXYGEN SPECIES WITH IMMUNOREGULATORY POTENTIAL

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CLASSICAL AND NEWLY RECOGNIZED SOURCES OF REACTIVE OXYGEN SPECIES IN THE IMMUNE SYSTEM

Aerobic organisms, which rely on 4-electron reduction of dioxygen (O_2) to water (H_2O) for generation of energy, constantly produce low levels of products of 1-,2- or 3-electron reduction of oxygen, i.e. superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot OH$), collectively termed reactive oxygen species (ROS), in various subcellular compartments, e.g. mitochondria, microsomes and cytoplasm. ROS from these sources are physiologically effectively counteracted within the cell, notably by the vitamins E and C, the enzymes superoxide dismutase and catalase and the glutathione — glutathione — peroxidase system; the consequence is that under normal circumstances aerobic cells do not release detectable ROS.

In contrast, certain cells of the immune system, namely phagocytes, have been known for some time to possess a special enzyme, NADPH oxidase, which upon activation yields superoxide by a one-electron reduction of dioxygen.[1] Recognized components of NADPH oxidase are a cytochrome *b* with low midpoint potential, cytochrome b_{-245},[2] and a 44–45 kDa flavoprotein, also detectable by its binding to an inhibitor of NADPH oxidase, di-phenylene-iodonium.[3] On activation of the enzyme, electrons are transferred from NADPH via the flavoprotein to the cytochrome *b*, which then serves as terminal electron donor to dioxygen.

Superoxide thus produced by activated phagocytes is involved in antimicrobial action of these cells, but may also be harmful to host cells, causing inflammation, tissue damage and mutagenesis. Though other cells of the immune system can also cause cytotoxicity and inflammation, so far ROS production by immunological cells has been observed only in phagocytes, not in lymphocytes. In several instances, claims of ROS production by non-phagocytic immune cells have been made, as for instance

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with natural killer cells.[4] However, ROS detected from preparations of such cells have later been shown to be generated by contaminating phagocytes.[5] Presently, the field of ROS production by cells other than professional phagocytes is experiencing renewed attention. Fibroblasts and mesangial cells which also participate in immune reactions have been recognized to form ROS after exposure to the inflammatory cytokines, tumor necrosis factor and interleukin-1.[6] Recently, another cell central to the immune system, namely the B lymphocyte, has been found to possess a superoxide generating system with unique features of triggering. In the following we shall summarize what is presently known about this B cell – associated superoxide generating system, which we have termed "B cell oxidase", and we shall present a hypothesis of the possible involvement of the B cell oxidase in immonoregulation.

THE SUPEROXIDE GENERATING SYSTEM OF EPSTEIN-BARR-VIRUS – TRANSFORMED HUMAN B LYMPHOCYTES (EBV-BLCL)

In contrast to the situation with natural killer cells, no function of B cells led researchers to suppose that it might be connected to ROS production. Rather, Volkman *et al.*[7] serendipitously observed that human EBV-transformed B lymphocyte cell lines derived from healthy individuals reduced nitroblue tetrazolium (NBT), and ferricytochrome C in an SOD-inhibitable fashion when exposed to the protein kinase C activating agent phorbol myristate acetate. In contrast, EBV-BLCL derived from patients with chronic granulomatous disease (CGD) did not show this property. Since CGD is a group of genetic disorders in which phagocytic NADPH oxidase is either non-functional or of low activity, they argued that the superoxide generating system of normal EBV-BLCL was the same as in phagocytes, NADPH oxidase. This notion was supported by biochemical studies of the B cell oxidase performed by our group, spectrophotometrically demonstrating the presence of cytochrome b_{-245}, and of a 45 kDa Di-phenylene-iodonium (DPI) – binding peptide believed to be the flavoprotein of NADPH oxidase, in EBV-BLCL capable of PMA-triggered superoxide production. Furthermore, DPI ($1–10 \times 10^{-6}$ M), but not KCN (up to 10^{-3} M), inhibited superoxide production of B lymphocytes.[8] Using a monoclonal antibody recognising an extracellularly accessible epitope of the small 22 kDa subunit of Cytochrome b_{-245} (mAb 7D5),[9] and a cDNA probe for the large subunit of cytochrome b_{-245}[10] we could further confirm presence of both components of cytochrome b_{-245} in EBV-BLCL.[11] Interestingly, Cytochrome b_{-245} components were also present in Burkitt lymphoma (BL) cell lines which did not produce superoxide when exposed to PMA up to 100 ng/ml, but could not be demonstrated in the T-lymphocyte tumor line Jurkat. In a recent study, our group reported superoxide generation from membrane preparations of PMA-triggered EBV-BLCL or BL triggered with high amounts of PMA (5 mg/ml) after addition of NAD(P)H, with NADPH being the preferred substrate. Interestingly, superoxide production of these membrane preparations was 10–20 × higher than that of intact cells, suggesting that in intact B cells an inhibitor regulates oxidase activity, which becomes detached or inactivated after cell disruption.[12] Such tight regulation would be expected for a superoxide generating system in a cell type that is destined for a relatively long lifespan and further proliferation.

NORMAL, NON-TRANSFORMED B CELLS PRODUCING SUPEROXIDE

Superoxide production by transformed or tumor B cell lines via NADPH oxidase as described above could reflect a property of their "natural counterparts", i.e. normal B cells. Alternatively, it could be due to a lineage-untypical aberrant expression of genes normally active only in phagocytes, caused by the transformation event. A number of facts argued against the latter possibility: So far, each EBV-BLCL investigated showed superoxide production, but no other phagocytic properties, indicating specific gene expression. Further, genes coding for NADPH oxidase are located on different chromosomes, as evidenced by the existence of autosomal and X-chromosomal forms of CGD. However, EBV genome integrates randomly into B cell DNA. Though a specific trans-activation of NADPH oxidase genes by EBV would be possible, it is an unlikely possibility. In fact, we recently demonstrated superoxide-mediated NBT reduction, and expression of cytochrome b_{-245} small subunit antigen on human B lymphocytes of tonsillar origin.[11] Quantitatively, the potential of tonsillar B cells to produce superoxide was estimated between 1–2 nMol/10^6 cells × hr, being in the same range as found with EBV-BLCL, but 50–100 times lower than that of maximally active human granulocytes. In view of the difficulty of removing phagocytes totally from lymphocyte preparations, it is therefore not surprising that superoxide production by B cells has not been recognized earlier.

Since the presence of a superoxide generating system on EBV-BLCL appears thus as a consequence of immortalisation of normal, non-transformed B lymphocytes possessing this property, EBV-BLCL can be utilised as model systems for the study of activation, and of biological significance of superoxide production by B cells. However, as to the latter, cell lines may not be optimal objects of examination because long-term culture in the presence of atmospheric oxygen can be expected to result in the selection of cells with enhanced anti-oxidative defenses.

MODES OF ACTIVATION OF B CELL OXIDASE

Phorbol ester was the first stimulus reported to trigger superoxide production by EBV-BLCL. Later, we have found that like the phagocytic NADPH oxidase, the B cell oxidase can also be triggered by the G-protein-activating agent sodium fluoride.[13] In contrast, other substances which are very effective stimuli for superoxide production in phagocytic cells, i.e. latex particles and calcium ionophore, did not stimulate superoxide production in EBV-BLCL. Thus, a physiological stimulus for the B cell oxidase was first lacking. However, our group later showed that both in EBV-BLCL and in normal tonsillar B cells, cross-linking of the B cell's receptor for antigen, surface immunoglobulin, by protein A or anti-Ig antibody also triggers the B cell oxidase.[8,11] In line with this, we recently found that specific antigen also triggers the B cell oxidase. Interestingly, to be stimulatory, antigen had to be present either as solid-phase immobilised "poly-antigen" or in form of a highly antigen-derivatized soluble carrier molecule. In contrast, antigen in soluble mono-or oligomeric form was ineffective and even inhibited the solid phase – antigen triggered response (our unpublished results). It appears thus likely that *in vivo*, repeating antigenic epitopes present on macromolecules or on cellular surfaces can serve as stimuli for the oxidase

of B cells with appropriate specificity. Insofar, activation of the B cell oxidase by solid-phase antigen bears resemblance to the situation of phagocytic cells exposed to stimuli immobilised on solid phases too large to be engulfed ("frustrated phagocytosis"). For example, with neutrophils exposed to immunoglobulin G on a glomerular basement membrane matrix, activity of the neutrophil oxidase was found restricted to those areas of the cell membrane in direct contact with the solid phase.[14] It has been suggested that under such conditions, reactive oxygen species are mainly liberated into, and effective within, this relatively secluded microenvironment. Thus, B cells presumably constitute a very specific and presumably locally active source of ROS *in vivo*, in contrast to the mostly unspecifically activated phagocytes.

IMMUNOLOGICAL CONSEQUENCES OF ROS GENERATION

In view of the oxidizing capacity of ROS towards practically all types of biomolecules – proteins, polysaccharides and nucleic acids – it is perhaps not surprising that various immune phenomena are subject to the influence of reactive oxygen species. Interestingly, however, both positive and negative effects have been described in various *in vitro* systems. A few examples may illustrate these points: On the positive side, H_2O_2 induces the expression of interferon-gamma by natural killer cells;[15] and ROS facilitate the release of tumor necrosis factor in response to bacterial lipopolysaccaride both *in vitro* and *in vivo*.[16] Oxidation of the precursor protein is also necessary for the development of immuno-suppressive activity of the lymphokine soluble immune response suppressor (SIRS).[17] T lymphocyte activation and proliferation also appears to contain a ROS-dependent step.[18] Further, a link between antigen presentation and reactive oxygen species has been suggested from a quantitative defect of *in vitro* antigen presentation in patients with CGD, who are unable to produce superoxide via NADPH oxidase.[19] In fact, oxidation-hydroxylation via cytochrome P-450 is a common pathway by which the body metabolises foreign material; this metabolism may lead to altered immunogenicity of the metabolised as opposed to the original compound. It has thus been reasoned that antigens might also be modified by oxidants generated from antigen-presenting cells like macrophages and B cells.[20]

On the negative side, hydrogen peroxide released from monocytes/macrophages can inhibit production of IL-2 and proliferation of T lymphocytes, and immunoglobulin synthesis by B lymphocytes.[22,22,23] Thus, "net effects" of reactive oxygen species on immune phenomena appear different for each situation, probably dependent on the amount, time course, localisation and predominant type of ROS generated.

HYPOTHESIS: POTENTIAL SIGNIFICANCE OF B LYMPHOCYTE-DERIVED ROS FOR IMMUNE REACTIONS

Combining the current knowledge of B cell physiology with the recognition of B cells as potential sources of reactive oxygen species, certain phenomena in particular appear candidates for an involvement of B cell-derived oxidants. For instance, high doses of antigen suppress B cell function;[24] this might be related to triggering of the oxidase by multivalent cross-linkage of the antigen receptor, surface immunoglobulin.

Further, B cells have recently been found capable of lysing certain tumor cells and virally infected cells.[25,26] Though this may be due to release of lymphotoxin, it is also possible that oxidants derived from B cells may be involved. Finally, B cells show the phenomenon of somatic mutation of immunoglobulin genes, leading to the generation of mutated immunoglobulins some of which have higher affinity for antigen than the "parent" antibody.[27] So far, there is no explanation of what might be the mechanism of these seemingly random mutations. In view of the capability of B cells to produce potentially mutagenic oxidants after encounter with antigen, it is appealing to speculate that these oxidants, perhaps "targeted" by heavy metal ions, may be responsible for the observed mutations and thus fulfill an important function in the generation of antibody diversity. Regardless of the fate such hypotheses will find when tested experimentally, the recognition of B lymphocytes as a source of antigen-triggerable, and thus highly specific, clonally and probably also locally restricted, fluxes of ROS provides a rationale for the concept that B cell – derived ROS may be involved in B cell physiology and pathology.

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