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REVIEW

Chronic granulomatous disease: recent advances in pathophysiology and treatment

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ABSTRACT

Chronic granulomatous disease (CGD) was characterised half a century ago as a primary immunodeficiency disorder of phagocytic cells resulting in failure to kill a specific spectrum of bacteria and fungi and in concomitant hyperinflammation with widespread tissue granuloma formation. CGD now comprises five genetic defects, each impairing one of five essential subunits of the phagocyte NADPH oxidase generating reactive oxygen species. In the past few years CGD has lead to a new understanding of the importance of phagocyte oxygen metabolism for intra- and extracellular host defence and for resolution of the concomitant inflammatory process. In a not too distant future, this may help to tailor novel pharmacological and cellular interventions to the requirements of individual patients.

This review covers recent advances in the pathophysiology of CGD and outlines today's clinical presentation as well as the basic principles for treatment of this relatively rare genetic disease. 'Fatal' granulomatous disease 50 years later has become a chronic inflammatory disorder with a median survival of 30 years and is of interest to both paediatricians and internists.

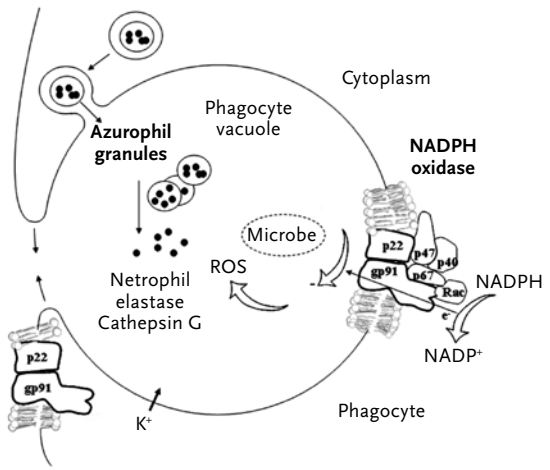
KEYWORDS

Chronic granulomatous disease, NADPH oxidase deficiency, microbial killing defect, hyperinflammation, stem cell transplantation, gene therapy

INTRODUCTION

Chronic granulomatous disease (CGD) is a group of five genetic disorders of the phagocyte (neutrophil, monocyte, macrophage, eosinophil) nicotinamide adenine dinucleotide phosphate (NADPH) *oxidase complex* generating reactive oxygen species (ROS) in response to physiological stimuli such as the phagocytosis of microbes.[1,2] The catalytic core of the phagocyte NADPH oxidase (phox) is *gp91phox*, a phagocyte-specific transmembrane glycoprotein with an apparent molecular mass of 91kDa, recently renamed NOX2.[3] Gp91phox/NOX2 transports electrons from cytosolic NADPH via flavin adenine dinucleotide (FAD), and two haemes onto molecular oxygen, which is then converted into superoxide anion and subsequently to several ROS (e.g. the highly diffusible hydrogen peroxide and hypochlorous acid) (*figure 1*). In the cell membrane NOX2 is stabilised by *p22phox*, which also serves as an anchoring site for three regulatory proteins: *p47phox, p67phox* and *p40phox*. The last three form a complex in the cytoplasm of resting phagocytes, which is translocated en bloc to the endocytosed cell membrane (the phagocytic vacuole

Figure 1. *Phagosome formation and oxidative killing of microbes by phagocytic cells*



ROS = reactive oxygen species

or phagosome) during phagocytosis. A small cytosolic GTPase, *Rac*, is also activated, translocates and induces a crucial conformational change within p67, needed to activate NOX2. p47 and p40 serve as adaptor molecules.
Mutations in all of the five structural genes of the NADPH oxidase complex have been found to cause CGD with an overall prevalence of 1:250,000. X-linked defects in NOX2 account for about 70% of cases, autosomal-recessive defects in p47 for about 20% and the remainder for the very rare p22 and p67 defects.[4,5] Last year a single p40phox deficient CGD patient was identified.[6] Clinically the NOX2 deficient form of CGD runs a more severe course than the p47 deficient form, with earlier presentation and earlier death.[4,5]
A provisional diagnosis of CGD is made by a DHR assay using flow cytometry or by nitroblue tetrazolium (NBT) using light microscopy. DHR (dihydrorhodamine-1, 2, 3) freely enters the phagocytes and is oxidised intracellularly to rhodamine-1, 2, 3 by diffusible H_2O_2 after phagocyte stimulation.[7] Since the assay relies on endogenous myeloperoxidase (MPO), it will give a false-negative DHR result in complete MPO deficiency which can be misinterpreted as variant NADPH oxidase deficiency (CGD).[8] NBT is a yellow dye that is co-phagocytosed with microbial particles and reduced by superoxide to blue, insoluble formazan, which cannot leave the phagosomes.[7] In contrast to the objective DHR assay, the NBT test is subjective being based on microscopic inspection of a limited number of cells, classical CGD patients showing no formazan formation. Variant CGD with residual ROS production may be missed in the NBT test as it manifests as faint blue staining.[9] X-linked carriers of NOX2 deficiency have a mosaic pattern of normal and defective neutrophils on oxidative testing by either DHR or NBT, ranging in most cases from 20 to 80% oxidase positive cells. It must be kept in mind, however, that up to one-third of x-linked defects arise from new mutations in germ-line cells of the mother and will not be present in her somatic cells.
The provisional diagnosis of CGD should always be checked by a specialist centre, where a definitive diagnosis can be given by immunoblotting for the components of NADPH-oxidase and by DNA-based molecular techniques. Molecular determination of the disease-causing mutation(s) is required before genetic counselling, before prenatal or preimplantation diagnosis and before gene therapy.

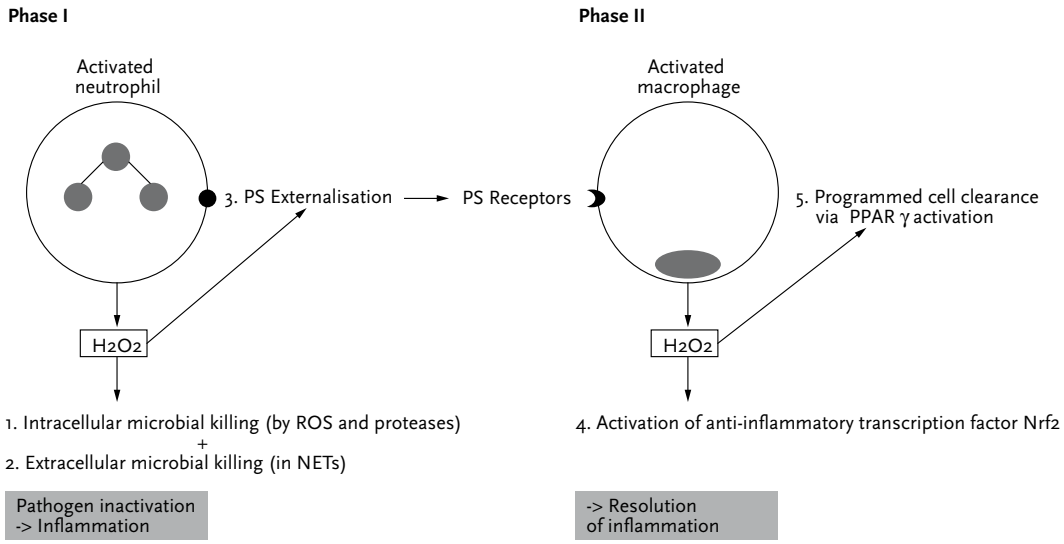
ADVANCES IN PATHOPHYSIOLOGY

Knowledge gained on the mechanisms by which NADPH oxidase normally kills microbes and resolves inflammation has broad relevance to understanding host-pathogen interactions (*figure 2*).

Deficient antimicrobial defence

Some oxygen metabolites generated by NADPH oxidase activation show direct cytotoxicity and antimicrobial properties.[10] NADPH oxidase can also mediate intracellular host defence indirectly by activation of microbicidal granule *proteases* in neutrophils as recently shown.[11] The influx of electrons into phagosomes is compensated

Figure 2. *Phagocyte NADPH-oxidase functions in host defence and inflammation*



NETs = neutrophil extracellular traps; PS = phosphatidylserine

by cation fluxes across the phagosomal membrane to maintain electrogenic neutrality. The increased ionic strength leads to solubilisation and activation of granule proteases, e.g. elastase and cathepsin G, bound to an anionic proteoglycan matrix stored in primary granules. In CGD neutrophils the granule matrix is not resolved after fusion of granules with the phagosome.

Neutrophils also release cytosolic and granule proteins as well as chromatine (DNA/histones), which mix to form neutrophil extracellular traps (NETs). These NETs bind and kill bacteria[12] and target fungi.[13] CGD neutrophils are deficient in NET formation,[14] which was reversed in one patient by gene therapy and was accompanied by resolution of his therapy-refractory pulmonary aspergillosis.[15] Current studies aim at understanding the relative contributions to antimicrobial host defence of each of the three ROS-dependent killing mechanisms: NADPH oxidase-generated ROS *vs* protease activation *vs* NET formation.

Excessive inflammation

There is new strong clinical and experimental evidence that the NADPH oxidase is critical for downregulation of inflammation. The recently recognised syndrome of 'mulch pneumonitis' in CGD patients exemplifies this role.[16] Two to three days after spreading mulch or clearing mouldy leaves (and inhaling *Aspergillus* spores) fever and dyspnoea manifest with diffuse interstitial infiltrates on chest radiographs and hypoxia. Successful treatment requires both antifungals and steroids, the latter to prevent extensive *Aspergillus*-triggered inflammation.

In CGD mice intratracheal instillation of zymosan (a fungal cell wall product of beta-glucans) elicits progressive pyogranulomata. Recent studies revealed a crucial role for a ROS-sensitive anti-inflammatory transcription factor, Nrf2. Consistent with these findings mononuclear blood cells from CGD patients stimulated by zymosan showed reduced Nrf2 activity.[17] In agreement with a proinflammatory state, monocytes from CGD patients reveal *increased* inflammasome activity as manifested by activation of caspase 1, followed by Il-1β production and release.[18,19]

Recent studies suggest an additional role of the NADPH oxidase in the process of macrophage mediated clearance of activated and infected neutrophils. Externalisation of the anionic phospolipid, phosphatidylserine (PS), on the neutrophil surface is recognised by PS receptors in macrophages and facilitates the uptake and degradation of such neutrophils.[20] CGD neutrophils are defective in ROS-dependent exposure of PS on the cell surface. In addition CGD macrophages have a reduced capacity for uptake of PS-positive target cells, thus impairing resolution of the inflammatory process.[21] PPARγ is known to upregulate proteins involved in both apoptotic cell recognition and digestion and has been found deficient in CGD macrophages. Pharmacological activation of PPARγ by proglitazone in CGD mice normalised uptake of CGD neutrophils by CGD macrophages. Proglitazone may thus be effective in the clinical treatment of CGD inflammation[22] and needs to be tested further.

Currently glucocorticoids are used as main anti-inflammatory agents in CGD. They prevent tumour necrosis factor-alpha-dependent multinucleated giant cell formation[23] and promote non-phlogistic phagocytosis of activated neutrophils by macrophages.[24] The ongoing molecular dissection of Nrf2 and PPARγ activation in CGD may hopefully yield novel targets for tailored pharmacological interventions in the near future, avoiding many of the steroid side effects.

RECURRENT INFECTIONS

Skin, lymph nodes, lung, and liver are the most frequent sites of infection in CGD. In North America and Europe five main groups of organisms persisting inside CGD neutrophils predominate:[5] *Staphylococcus aureus* (lymphadenitis, liver abscess), *Burkholderia* complex (necrotising pneumonia +/- sepsis), *Serratia marcescens* (sepsis +/- skin ulcers and osteomyelitis), *Nocardia and Aspergillus* spp (pneumonia +/- dissemination to brain and bone). The infections arise mostly from inescapable environmental exposure and intermittent compliance with long-term antimicrobial prophylaxis.

In other parts of the world different microbial agents predominate. Mycobacterial infections (due to *BCG* and *M. tuberculosis)* in CGD patients have been reported from China, Iran and Latin America.[25,26] Patients, however, develop severe localised (not disseminated) BCG infection and pulmonary (not miliary) tuberculosis. Infection-associated haemophagocytic syndrome in CGD patients triggered by *Leishmania* has been observed in the Mediterranean region.[27]

Recently, two additional chronic infections due to fastidious organisms were described in North American and European patients, requiring combined antibiotic therapy and surgery: *Granulibacter bethesdensis*, a gram-negative rod growing on charcoal yeast extract at 35° causing chronic multifocal necrotising lymphadenitis and requiring long-term ceftriaxone therapy,[28] and *Actinomyces* spp, gram-positive rods, growing anaerobically on sheep blood agar at 37° causing severe chronic actinomycosis and necessitating long-term penicillin G/V therapy.[29]

Diagnostic work-up of infections in CGD requires a vigorous microbiological diagnosis with full help from a specialist microbiology laboratory. Needle biopsies, ribosomal DNA-PCR analyses and susceptibility testing of difficult-to-grow organisms are important steps towards a tailored antimicrobial therapy. Whole body PET/CT scans

can be useful to localise occult infections for biopsy and to exclude dissemination.[30]

Prevention and treatment of infections

Common sense measures in reducing exposure to infectious agents can be downloaded from www.cgd.org.uk. Pulmonary infections can be prevented by avoiding sources of *Aspergillus* spores (e.g. farms, mulch, construction sites) and refraining from smoking. Patients should receive all routine immunisations, except BCG.

The cornerstone of clinical care is lifelong antibiotic and antifungal prophylaxis. Drugs of choice are the lipophilic co-trimoxazole (at 6 mg/kg/day of trimethoprim; during pregnancy replaced by cefuroxime) and itraconazole (at 5 mg/kg/day oral solution). Retrospective studies support long-term co-trimoxazole prophylaxis,[31,32] and a randomised, double-blind placebo-controlled study justifies routine administration of itraconazole in CGD.[33] Interferon-gamma prophylaxis is offered by most European physicians only in selected CGD cases. A small subgroup of variant X-CGD patients with splice site mutations has been shown to be responsive to interferon stimulation[34,35] through improved splicing. A significant clinical efficacy in preventing aspergillosis, however, has not been demonstrated yet.[36] In addition, the drug is expensive and requires repeated injections (2 x 50 ug/m^2/week subcutaneously).

Before culture results are available, empiric antibiotic therapy has to be based on the most likely infectious agents expected. Antibiotics should cover a broad range of bacteria including *S. aureus*, *Burkholderia*, *S. marcescens*, and *Nocardia*. Oral ciprofloxacin and intravenous meropenem are useful first-line agents. A course of oral ciprofloxacin can also be taken as reserve on holidays. In addition co-trimoxazole should be continued at a double dose (12 mg/kg/day trimethoprim) to compensate for possible intermittent compliance. In case of pneumonia, voriconazole needs to be added as antifungal agent (in children at 12 mg/kg/day). As infections often respond slowly, intravenous treatment must be followed by prolonged oral therapy. Treatment must be further extended if special organisms are isolated (eg. *Nocardia* spp and *Aspergillus* spp). A novel antibiotic, Linezolid, has proven effective second to high-dose co-trimoxazole (at 20 mg/kg/day trimethoprim) in nocardiosis with excellent penetration of the cerebrospinal fluid.[37] Posaconazole is effective salvage therapy against a broad spectrum of invasive fungal infections, including the difficult-to-treat infections of the central nervous system.[38]

Surgical procedures in CGD comprise drainage of abscesses, excision of a consolidated focal infection in lung or liver and relief of obstructions (e.g. hydronephrosis). Wounds and surgical sites in CGD heal very slowly and may form fistulas. Liver abscesses in CGD are dense and caseous. This is why larger liver abscesses require surgical excision and drainage in addition to a one- to two-month course of antibiotic therapy.[39] When surgery is contraindicated experimental approaches can be tried: percutaneous radiofrequency thermal ablation as used for treatment of liver cancer[40] or steroids at 1 mg/kg/day in addition to the antibiotic therapy.[41]

With the advent of potent new antifungal drugs the use of white cell transfusions has decreased considerably. Alloimmunisation to HLA class I antigens and transfer of cytomegalovirus (CMV) by infected neutrophils has complicated subsequent allogeneic stem cell transplantation. Therefore, white cell transfusions should now be reserved as a last resort.

INFLAMMATORY COMPLICATIONS

Persistent inflammation also occurs independently of infection. The inflammatory complications of CGD are most prominent in the gastrointestinal and urinary tracts. Gastric outlet obstruction is common.[42] About one third of patients are affected by granulomatous colitis mimicking Crohn's disease.[43,44] In the urinary tract the most common manifestation is inflammatory cystitis which can lead to obstruction of ureteric orifices and cause hydronephrosis. Imaging findings of infections and inflammatory complications often have unique features that can help to suggest a diagnosis of CGD.[45]

Treatment of exuberant inflammation

Cautious use of immunosuppressive therapy, namely corticosteroids, is required for acute granulomatous exacerbation in the lung, the bowel and urinary tract as well as for inflammatory bowel disease (IBD). Invasive lung aspergillosis and nocardiosis profit from initial addition of steroids (1 mg/kg/day for three days, then taper). Granulomatous cystitis quickly responds to corticosteroids (e.g. 0.5 to 1 mg/kg/day prednisone for the first week, to be tapered over six weeks). First-line therapy for IBD (*table 1*) in severe cases is prednisone (e.g. 1 mg/kg/day) with gradual tapering over several months. If high-dose steroids are administrated in the long term, antifungal prophylaxis should be switched from itraconazole to voriconazole, since itraconazole is a strong inhibitor of CYP3A4 increasing steroid levels threefold by blocking their degradation.[46] Faecal calprotectin is a good measure for follow-up of colitis activity.[47] Anti-TNF drugs (e.g. Remicade®) may be administered short term for remission induction in steroid-refractory patients. Long-term anti-TNF therapy combined with steroids however is contraindicated because of high risk of infections in an already genetically

Table 1. *CGD: drugs for treatment of granulotomatous colitis*

	Mildly/moderately active	Severely active	Perianal fistulas***
Topical treatments			
sulfasalazine oral (40-50 mg/kg/day)	+ (induction ± maintenance)	-	-
Systemic treatments			
prednisone oral (1 mg/kg/day, then taper)	+ (induction)		
iv. (1 mg/kg/day, then slow taper	-	+ (induction ± maintenance)	-
infliximab (5 mg/kg at 0, 2 6 weeks)*	-	+ (induction, if steroid refractory)	+ (induction)
azathioprine (2.5 mg/kg/day)**	-	+ (maintenance), if steroid dependent or refractory)	+ (maintenance)

***in CGD not for maintenance; **slow onset of action (3-4 mo); ***add metronidazole/ciprofloxacine.**

immunodeficient patient. Thalidomide may also be used as a successful treatment for refractory CGD colitis.[48] Steroid-dependent or refractory colitis can be cured by stem cell transplantation with rapid induction of remission (within two months),[49,50] avoiding the need for extensive colectomy and subsequent anastomosis complications.

CURE OF THE DISEASE

Haematopoietic stem cell transplantation

Conventional myeloablative marrow conditioning followed by transplantation of unmodified haematopoietic stem cells is a definite cure for CGD. In 2002 a European collaborative study reported the outcome of 27 mostly paediatric CGD patients receiving a busulfan-based regimen followed by an human leucocyte antigen (HLA) genoidentical marrow graft from a sibling donor (MSD).[49] Severe side effects from haematopoietic stem cell transplantation (HSCT), namely graft-*versus*-host disease and inflammatory flare-up, were exclusively seen in a subgroup of nine patients with ongoing infection, mainly aspergillosis. Overall survival was 85%, with 81% of patients cured. Most cured patients had >95% circulating donor myeloid cells. Pre-existing infections and chronic inflammatory lesions cleared in all engrafted survivors. Even children with severe lung restriction improved their lung function, albeit slowly.

These favourable results after myeloablation have recently been extended to matched unrelated donor (MUD) transplants. In a series from Ulm seven of nine MUD recipients are survivors[51] and 18 of 20 transplanted in Newcastle (from 10 MSD and 10 MUD) survived with most patients achieving normal neutrophil function, remission of colitis and catch-up-growth.[50]

The decision for or against HSCT should be made early in life based on the individual clinical course. HSCT may be most useful in patients with recurrent serious infections despite correct antimicrobial prophylaxis or with severe steroid-dependent inflammatory complications provided an HLA identical stem cell donor is available.[52]

Two other recent advances are noteworthy. The first is the development of a reduced intensity conditioning regimen (RIC) for adults and paediatric CGD patients over six years of age. RIC-HSCT using busulfan (8 to 10 mg/kg), adjusted with busulfan kinetics, fludarabine (180 mg/m^2), ATG-Fresenius (40 mg/kg) and HLA-matched donors (MSD=5, MUD=3) was performed in eight high-risk CGD patients in Zurich. All engrafted with full donor chimerism, without graft-*versus*-host disease and with resolution of active infections and inflammatory foci, except for one adult patient who had received a CMV negative MUD and died on day +150 from CMV pneumonitis.[52,53] RIC with subsequent HSCT is thus a promising treatment modality for fragile CGD patients with intractable infection or inflammation.

Second, in the era of pre-implantation genetic diagnosis, saviour siblings can be selected at a very early embryonic (8 cell) stage to screen for CGD and to determine HLA type, and thus can be chosen as potential umbilical cord blood donors for an affected older sibling lacking an HLA-identical stem cell donor. As the probability of a successful pregnancy, even in the most experienced *in vitro* fertilisation centres, is low (10%), and the treatment option requires the firm wish of the parents to have another healthy child, this treatment modality has to be approached with reserve and is not allowed in all countries.[54,55]

Stem cell gene therapy

Gene therapy for restoration of the NADPH oxidase is the ultimate goal. Experimental gene therapy for CGD in selected patients with very poor performance status has recently been undertaken in Frankfurt, London, Zurich, Seoul and the USA (at NIH) in 12 patients.[56-58] A transitory beneficial effect on pre-existing infections similar to the clinical response known of several white

cell transfusions from healthy donors has been observed in all. A major obstacle however remained: the lack of selective growth advantage of gene transduced cells. Despite submyeloablative chemotherapy long-term engraftment was only observed in three out of the 12 patients.[58] In these three patients limited expansion of gene-corrected cells was seen due to activating retroviral insertions in the MDS1/Evi1 oncogene. These three patients later developed a myelodysplastic syndrome with monosomy 7 and two received a myeloablative allogeneic (MUD) rescue transplant.[59] For safety reasons a new generation, self-inactivating (SIN) vector lacking the potent retroviral enhancer elements and showing much less transactivation potential has been developed. Transgene expression within this vector is driven by a myelospecific, cellular promoter, thus further reducing the probability of oncogene activation at the stem cell level.[58]

In summary a transitory gene therapy approach to CGD has finally become feasible to overcome life-threatening, therapy-resistant infections. Permanent cure of CGD by gene therapy, however, remains a more distant goal.

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