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Acknowledgements
Research in the laboratories of N.A. and S.E.H. was supported by several grants from the Department of Biotechnology, Ministry of Science & Technology, Government of India. Research in the laboratories of U.D. and J.H. was supported by grants from the German Research Foundation (DFG-79, TP A1), the European Community (European virtual institute for functional genomics of bacterial pathogen; CEE LSBB- CT-2005-512,061) and the Bavarian Research Foundation.

DATABASES
Entrez Genome Project: http://www.ncbi.nlm.nih.gov/ entrez/query.fcgi?db=genomic
Entrez Plasmid Project: Mycobacterium avium
Mycobacterium bovis | Mycobacterium leprae | Mycobacterium marinum | Mycobacterium smegmatis | Mycobacterium tuberculosis | Mycobacterium ulcerans | Yersinia pestis | Yersinia pseudotuberculosis

FURTHER INFORMATION
Jörg Hacker and Ulrich Dobrindt’s homepage: http://www.infektionsforschung.uni-wuerzburg.de/
Niyaz Ahmed’s homepage: http://www.pathogen-evolution.org
Seyed E. Hasnain’s homepage: http://www.tiuinp.org/hasnain.html
Institute Pasteur (Collibri database): http://genolist.pasteur.fr/Collibri/
Institute Pasteur (PyloriGene database): http://www.genolist.pasteur.fr/PyloriGene/
International Society for Genomic and Evolutionary J. Craig Venter Institute (comprehensive microbial resource): http://www.ncbi.nlm.nih.gov/crm/research/projects/cm/
MIBI–VNTRplus: http://www.mibiplus.org/
Wellcome Trust Sanger Institute (pathogen sequencing unit): http://www.sanger.ac.uk/Projects/Pathogens/

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OPINION

Searching for the cause of Kawasaki disease — cytoplasmic inclusion bodies provide new insight

Anne H. Rowley, Susan C. Baker, Jan M. Orenstein and Stanford T. Shulman

Abstract | Kawasaki disease (KD) has emerged as the most common cause of acquired heart disease in children in the developed world. The cause of KD remains unknown, although an as-yet unidentified infectious agent might be responsible. By determining the causative agent, we can improve diagnosis, therapy and prevention of KD. Recently, identification of an antigen-driven IgA response that was directed at cytoplasmic inclusion bodies in KD tissues has provided new insights that could unlock the mysteries of KD.

Kawasaki disease (KD) is an acute childhood illness that usually affects previously healthy infants and children. The disease is manifested by a high spiking fever and, in classic cases, four of five additional features: rash; red eyes; red lips and mouth; swollen and red hands and feet; and swollen glands in the neck. These symptoms resolve spontaneously within 1–3 weeks, or sooner after treatment with intravenous gammaglobulin and aspirin. However, inflammation of medium-sized arteries throughout the body, particularly of the coronary arteries, can occur during
the acute illness and result in coronary-artery aneurysms in 25–30% of untreated patients\(^1\)\(^2\)\(^3\). In severe cases, KD leads to heart attacks, coronary-artery-aneurysm rupture and/or sudden death\(^4\)\(^5\). Affected children can require interventions, such as angioplasty or stent placement, coronary-artery-bypass surgery or, rarely, heart transplantation\(^6\)\(^7\)\(^8\). Because the features of KD resemble those of other febrile childhood illnesses and as there is no specific diagnostic test for KD, diagnosis can be delayed or never established, which results in a higher likelihood that coronary-artery abnormalities will develop\(^9\). Incomplete clinical presentations of KD, in which children present with fever but fewer than four of the other classic features, make diagnosis especially difficult\(^10\). Although treatment with intravenous gammaglobulin and aspirin is an effective therapy for KD, its mechanism of action is unknown, not all children respond and the optimal treatment for children with refractory KD remains unclear\(^11\)\(^12\)\(^13\)\(^14\). Identification of the aetiology of KD would greatly enhance efforts to develop a diagnostic test, improve therapy and prevent KD.

Clinical features of KD that support an infectious cause include: abrupt onset of symptoms that are compatible with infection, and resolution of the illness in 1–3 weeks, even without treatment and usually without recurrence. The young age group that is affected, the winter–spring predominance of cases in non-tropical climates and the existence of epidemics or clusters of cases that spread in a wave-like manner throughout a community also suggest an infectious cause\(^15\). In the 40 years since Tomisaku Kawasaki initially described the clinical features of KD\(^16\), many possible aetiological agents have been suggested (TABLE 1), but none have been confirmed by subsequent study. Studies of KD aetiology and pathogenesis are fraught with difficulties. Accessing the most important target tissue of the disease, the coronary artery, for aetiological and pathogenic studies is not possible in living patients. As KD is an illness of small children, there are also ethical constraints on obtaining biopsy samples for research studies from lymph nodes and other tissues. So far, it has not been possible to reproduce the disease in an animal model by injecting blood, body fluids or tissue samples from acutely ill patients.

By examining tissue samples from fatal cases of KD, recent progress has been made in understanding KD aetiology and pathogenesis\(^17\)\(^18\)\(^19\)\(^20\). These studies revealed that oligoclonal IgA plasma cells infiltrate inflamed tissues, including coronary arteries\(^17\)\(^18\). Synthetic versions of these oligoclonal KD antibodies bind to an antigen in acute-KD-inflamed ciliated bronchial epithelium\(^21\). Light and electron microscopy studies have demonstrated that the antigen is localized to cytoplasmic inclusion bodies in tissues inflamed by acute KD\(^22\). In this Opinion, we discuss pathological and immunological findings of acute KD, with an emphasis on the IgA immune response and its antigenic targets, and on the importance of macrophages and their secreted factors in the pathogenesis of coronary-artery-aneurysm formation. Although other theories of KD aetiology will be discussed, our primary goal is to highlight a new pathway of discovery in KD research: the identification of IgA plasma cells in tissues inflamed by KD and the subsequent identification of viral-like cytoplasmic inclusion bodies in tissues inflamed by KD. The proposed pathogenesis of KD described below and illustrated in FIG. 1 represents the opinion of the authors, which was formed on the basis of these findings and the well-described pathological features of KD.

**History of KD aetiology studies**

Clinical, epidemiological and pathological studies of KD that were performed in Japan in the 1970s and 1980s quickly led to the hypothesis that an infectious agent is the cause of KD. A ubiquitous agent is suspected because of the rarity of KD in infants under 2 months of age (young infants could be protected by passive maternal antibodies) and in adults (who could have experienced asymptomatic

| Postulated agent | Proposed pathogenesis | Current status | Refs |
|------------------|-----------------------|----------------|------|
| Mercury          | Direct toxic effect   | Lack of supporting evidence | 98   |
| Rickettsia-like agent | Infection of macrophages and endothelial cells | Lack of supporting evidence | 36   |
| Propionibacterium acnes | Infection of macrophages and endothelial cells | Lack of supporting evidence | 35   |
| Rug shampoo      | Aerosolization of mites or a direct toxic effect | Lack of supporting evidence | 37–39 |
| Leptospira spp.  | Infection of endothelial cells | Lack of supporting evidence | 99   |
| Streptococcus sanguis | Infection or toxin effect | Lack of supporting evidence | 100  |
| Retrovirus       | Infection of lymphocytes | Lack of supporting evidence | 40–43 |
| Epstein–Barr virus or cytomegalovirus | Infection of various cell types | Lack of supporting evidence | 101,102 |
| Toxic shock syndrome toxin 1 (TSST1) | Superantigen-induced immune response | Not confirmed by follow-up studies | 44–46 |
| Bacterial toxin other than TSST1 | Superantigen-induced immune response | Lack of supporting evidence; still under investigation | 72–74 |
| CoronavirusNL-63 | None                   | Not confirmed by follow-up studies | 47–49 |
| Human bocavirus  | None                   | Reported by one group; currently unconfirmed | 50   |
| Previously unrecognized persistent RNA virus | Infection of targeted cells with antigen-driven immune response; cytoplasmic inclusion bodies are formed and can persist | Under investigation | 17–22, 87 |

**Table 1 | Aetiological agents postulated for Kawasaki disease**
infection during childhood), and because most cases present during the first or second year of life (when susceptibility to most ubiquitous agents is highest)\textsuperscript{15,21,24}. KD affects all ethnic and racial groups worldwide, but the markedly higher attack rate of KD in children of Asian ethnicity suggests a genetic predisposition to symptomatic infection; an increased attack rate is also observed in Japanese–American children who have a Western diet and lifestyle\textsuperscript{25}. By the age of 5, 1 in 150 Japanese children\textsuperscript{26}, 1 in 1,000 Black children from the United States\textsuperscript{27} and 1 in 2,000 Caucasian children from the United States\textsuperscript{27} will have developed KD. Siblings of patients with KD and children whose parents have a history of KD are at higher risk than the general population\textsuperscript{28,29}. Genetic susceptibility to KD is probably polygenic — polymorphisms in several genes that are related to an immune response, such as interleukin-4, chemokine receptor 5, chemokine (C-C motif) ligand 3-like 1 and inositol phosphate kinase C\textsuperscript{30–32}, have been implicated, which is compatible with an aetiology that is probably infectious. Genome-wide linkage analysis has revealed linkage of KD with chromosome 12q24, and multiple candidate genes are located in this chromosomal region\textsuperscript{33}.

Extensive culture and serological studies have not been successful\textsuperscript{25,34}, and none of the proposed agents have been confirmed\textsuperscript{35–50} (TABLE 1). It has been suggested that KD is caused by multiple aetiological agents, because various microorganisms have been isolated from individual patients with KD\textsuperscript{51,52}. Although several illnesses that are not associated with fever and primarily involve a one-organ system can be triggered by diverse infectious agents, such as Reye syndrome and haemolytic uraemic syndrome, there is no precedent for a multisystem febrile childhood illness with distinctive clinical features, such as KD, to be the result of multiple diverse aetiological agents. Some childhood febrile illnesses, such as polio, roseola and fifth disease, were suggested to be the result of multiple agents, but were later found to be due to a single infectious agent or a group of closely related infectious agents. Because KD is a common illness, it is more likely that KD occasionally coexists with other infections, without causal relationships.

The hypothesis that a bacterial toxin causes KD is still favoured by some investigators. This theory is based on clinical similarities between KD and staphylococcal or streptococcal toxin-mediated illnesses, such as the peeling of hands and feet and strawberry tongue, on the finding that many cytokines are upregulated in the serum of patients with acute KD\textsuperscript{53–55} and on reports of over-representation of particular T-lymphocyte-receptor V\textsubscript{B} families in the peripheral blood of some patients with acute KD.

Expansions of T lymphocytes that possess V\textsubscript{B}2 and V\textsubscript{B}8 (REFS 58–60), V\textsubscript{B}2 and V\textsubscript{B}6 (REF 61) or V\textsubscript{B}2 and V\textsubscript{B}5 (REF 62) during acute KD have been reported by some investigators, whereas others have not observed V\textsubscript{B} expansion during acute KD\textsuperscript{53–65}. Predominant V\textsubscript{B}–T-lymphocyte-receptor usage, even if present in patients with acute KD, does not necessarily implicate a superantigen or bacterial-toxin aetiology of KD. Restriction of V\textsubscript{B}–T-lymphocyte-receptor usage can result from conventional antigens, including those expressed by lymphocytic choriomeningitis virus\textsuperscript{66}, influenza virus\textsuperscript{67}, reovirus\textsuperscript{68}, herpes simplex virus\textsuperscript{69} and hepatitis C virus\textsuperscript{70}. Complementarity-determining region 3 (CDR3) size profiling and sequencing of the CDR3 regions of expanded V\textsubscript{B}–family members is probably the best way to determine whether an expansion is the result of a conventional antigen or a superantigen. In two studies that lacked CDR3 size profiling but included sequencing of PCR products, clonality was not observed in the one V\textsubscript{B} family that was sequenced from eight patients with KD or in the two V\textsubscript{B} families that were sequenced from one patient with KD. In another study in which CD4\textsuperscript{+} and CD8\textsuperscript{+} T lymphocytes from eight...
patients with acute KD were separated and Vβ families from each compartment were amplified by PCR, CDR3 size profiling and sequencing revealed clonal expansions of CD8+ T lymphocytes. Additional experiments that include separation of the CD4+ and CD8+ compartments and CDR3 size profiling and sequencing of expanded Vβ families might resolve these discrepancies.

Studies that have examined possible antibody responses to various bacterial superantigens in patients with KD are similarly conflicting. A study that implicated colonization of mucosal surfaces of patients with acute KD with toxic shock syndrome toxin 1–producing strains of Staphylococcus aureus as being aetiologically related to acute KD was not confirmed by subsequent study. To explain the multisystem nature of acute KD, a bacterial toxin would need to circulate in the bloodstream, but no bacterial toxin has been detected as yet in the peripheral blood of patients with KD.

An autoimmune mechanism of KD pathogenesis has also been proposed. The spontaneous resolution of KD and its generally non-recurring nature make this theory less attractive.

Recently, cytoplasmic inclusion bodies were identified in the ciliated bronchial epithelium of children with fatal acute KD. The presence of inclusion bodies in inflamed tissues during an acute illness such as KD is highly suggestive of an infection that is due to an intracellular pathogen, such as a virus. These inclusion bodies were identified using synthetic versions of IgA antibodies that are prevalent in the acute KD arterial wall, which provides strong support for their role in KD aetiology and pathogenesis.

Pathological findings in KD
KD is a systemic inflammatory disease that affects many organs and tissues. Ductal tissues and arterial tissues seem to be particularly targeted by the inflammatory process (FIG. 1). From a clinical perspective, the most important aspect of KD pathogenesis is inflammation of the medium-sized arteries, and particularly of the coronary arteries. Although endothelial cells are a major target of the disease process (FIGS 1, 2), they are clearly not the only target. Theories of KD aetiology that propose an endothelial cell antigen that is targeted by the immune system as the exclusive mechanism of disease pathogenesis fail to explain the presence of bronchitis, pancreatic and prostatic ductitis and other pathological features that are observed in autopsy studies of patients with KD, as well as the myocarditis that is noted in endomyocardial biopsies from living patients with KD (BOX 1). Infiltrating macrophages, T lymphocytes and cellular components of the arterial wall, such as myofibroblasts, are important in disease pathogenesis and might secrete a number of inflammatory mediators, enzymes and other molecules, such as vascular endothelial growth factor (VEGF), which contributes to vascular leakage and oedema (FIG. 2).

Immunological findings in acute KD
KD is an interesting example of an illness in which the distribution of inflammatory cell types in peripheral blood differs markedly from that in target tissues (TABLE 2). Therefore, studies that focus solely on peripheral blood could be misleading. Blood levels of many pro-inflammatory cytokines are elevated during acute KD, as might be expected in an illness that is manifested by prolonged fever and multiorgan-system inflammation.

Macrophages are particularly numerous in intimal and adventitial layers of
coronary-artery aneurysms. These macrophages are the likely source of matrix metalloproteinases (MMPs), such as MMP2 and MMP9, and other enzymes that can disrupt collagen and elastin fibres and weaken the arterial wall, thereby causing it to lose its structural integrity, after which it expands into an aneurysm (FIG. 2). VEGF and its receptor FLT1 are upregulated in endothelium, in vascular media and in macrophages in the arterial wall, and probably contribute to vascular permeability.

E-selectin and vascular cell adhesion molecule 1 are observed on perivascular (adventitial) new blood vessels in the coronary-artery-aneurysm wall, and may promote endothelial cell-inflammatory cell interactions and entry of macrophages and lymphocytes into arterial tissue. Years after the onset of KD, various growth factors, such as transforming growth factor-β1, platelet-derived growth factor-A (PDGF-A) and basic fibroblast growth factor (bFGF), are expressed in the arterial wall and could contribute to the neoangiogenesis and intimal proliferation that are often observed in autopsy studies of late-stage-KD deaths. VEGF and bFGF, and to a lesser extent PDGF-A, have been detected in inflammatory cells that infiltrate coronary aneurysms during acute KD, indicating that vascular remodelling and neoangiogenesis are ongoing even during the acute phase (FIG. 2).

Inflammatory cells in non-vascular tissues are generally also lymphocytes and large atypical mononuclear cells, macrophages and plasma cells. No purulence is noted in lymph nodes or other tissues, and neutrophils constitute only a small fraction of the inflammatory cells in inflamed KD tissues.

### The IgA immune response in acute KD

While screening an acute KD arterial-expression cDNA library with convalescent sera from patients with KD, it became apparent that many immunoglobulin-α clones were present in the library. Further study showed that IgA plasma-cell infiltration of the arterial wall was characteristic of acute KD, which prompted examination of non-vascular tissues. IgA plasma cells infiltrate the upper respiratory tract of patients with acute KD, often in a peribronchial distribution. Examination of other tissues inflamed by KD revealed IgA plasma-cell infiltration in the pancreas (during acute KD), especially around the pancreatic ducts and in the kidneys. Absolute numbers of IgA lymphocytes were decreased in peripheral blood in patients with acute KD compared with controls, which suggests that these cells may selectively enter target tissues of the disease in a manner that is similar to CD8+ T lymphocytes.

To determine whether IgA plasma cells in tissues inflamed by KD were oligoclonal (producing a restricted repertoire of antibodies, a characteristic feature of a specific, antigen-driven response) or polyclonal (producing a broad repertoire of antibodies without restriction of the response), the clonality of IgA genes in arterial tissue inflamed by acute KD was examined. Immunoglobulin heavy-chain-α genes that had been identified in the arterial wall of three children who had died of acute KD were sequenced in the CDR3 (antigen-binding) region, which revealed that each of the three patients had a restricted pattern of CDR3 usage that was characteristic of an antigen-driven response. In this study, unprocessed tissue was available from one of the three patients, and immunoglobulin-α genes from a primary cDNA library that had been made from this patient showed evidence of somatic mutation among the sequences, again supporting an antigen-driven response.
Table 2 Immunological features of acute Kawasaki disease

| Immune response                        | Peripheral blood                        | Arterial wall                                      |
|----------------------------------------|-----------------------------------------|---------------------------------------------------|
| Predominant inflammatory cell type     | Mature and immature neutrophil predominance | CD45RO+ T lymphocyte, macrophage (85,86), eosinophil (85,86), IgA plasma cell (17,18), and activated myeloid dendritic cell (86) predominance |
| Relative proportion of CD4+ and CD8+ T lymphocytes | CD4+ to CD8+ T lymphocyte ratio of ≥2; ratio of CD4+ to CD8+ T lymphocytes of ≥3.5 in the second week of illness in patients who develop coronary-artery aneurysms (87) | CD8+ T lymphocytes predominate over CD4+ T lymphocytes (80) |

Generation of synthetic KD antibodies.
To determine whether KD oligoclonal IgA antibodies were targeting a specific antigen (or antigens) in tissues inflamed by acute KD, α-heavy-chain immunoglobulin variable-region sequences that were present in arterial tissue inflamed by KD were cloned and a panel of monoclonal antibodies was generated (85). Antibodies made from immunoglobulin α-sequences that were more prevalent in the inflamed arterial tissue bound to acute-KD-ciliated bronchial epithelium much more strongly than antibodies that were made from less-prevalent sequences, which is consistent with an antigen-driven response (85).

KD antigen in cytoplasmic inclusion bodies.
The synthetic KD antibodies detected antigen in ciliated bronchial epithelium from patients with acute KD, but not in ciliated bronchial epithelium from infant controls (81). Antigen was also detected in a subset of macrophages in inflamed tissues from patients with acute KD (81). In bronchial epithelium, the intracytoplasmic spheroidal antigen that was detected had the morphological appearance of an inclusion body (Fig. 1). Haematoxylin and eosin-stained bronchial epithelium from patients with acute KD revealed corresponding amphophilic bodies, which suggests the presence of both nucleic acid and protein in the bodies (82). Transmission electron microscopy confirmed that the spheroidal bodies were homogeneous, granular, intracytoplasmic inclusion bodies (ICIs) (82). These studies showed that acute-stage-KD-ciliated bronchial epithelium contained ICIs that are consistent with aggregates of viral proteins and nucleic acids (82).

We propose that identification of these proteins and nucleic acids will lead to the identification of the KD aetiologic agent. Some examples of viruses that produce granular ICIs in infected tissues are the paramyxoviruses, reoviruses, poxviruses and filoviruses; the ICIs usually represent aggregates of viral proteins or nucleocapsids (86). Recent data indicate that ICIs are present in ciliated bronchial epithelium in approximately 85% of the children with KD who die in the first 2 months after the onset of KD and in approximately the same percentage of children who die more than 10 weeks after disease onset (when inflammation has generally subsided). Nucleic acid stains indicate that ICIs contain RNA, but not DNA. Although ICIs were not detected in the ciliated bronchial epithelium of control infants, they were present in the ciliated bronchial epithelium from approximately 25% of the control patients who were 9–84 years old. These older control patients had probably experienced asymptomatic infection with the ubiquitous KD agent; this high prevalence of ICIs in older control patients suggests that the KD agent can cause persistent infection. Taken together, these findings are consistent with a ubiquitous, persistent RNA virus as the aetiologic agent of KD (86).

We propose a model of KD pathogenesis in which the aetiologic agent of KD enters through the respiratory tract and infects ciliated bronchial epithelium, where it forms ICIs (Fig. 1). The agent might enter the bloodstream via macrophages, as a result of either infection or uptake by the cells that act as scavengers, before being carried to its target tissues, particularly coronary arteries, other arterial tissue and ductal tissues. Antigen-specific IgA plasma cells and CD8+ T lymphocytes infiltrate the targeted tissues to combat the pathogen and contain the infection, but the coronary arteries might be damaged by the products of activated macrophages and lymphocytes, such as MMPs. Alternatively, the infection could be controlled through gammaglobulin therapy by the provision of specific antibody, perhaps via antibody-mediated cellular cytotoxicity (87–90) (the mechanism for the dramatic clinical response of patients with Argentine haemorrhagic fever, an arenavirus infection to immune gammaglobulin).

The future
The limited availability of unfixed tissue samples from patients with KD has impeded our progress in understanding the aetiology and pathogenesis of the disease. Identification of the KD aetiologic agent will be accelerated by the placement of biopsy or autopsy tissues from patients with KD into an optimal cutting-temperature compound for storage at −70°C, into liquid nitrogen for rapid freezing with storage at −70°C and into glutaraldehyde and formalin. Ultrastructural analysis of ICIs in glutaraldehyde-fixed tissue and molecular studies of unfixed tissues will facilitate rapid progress. The new finding that ICIs are present in 25% of normal control patients might make more tissue available for research. The goal of these studies is to identify the causative agent of KD so that a diagnostic test and better therapies can be developed. Additional research on the genetics of KD could enable the identification of patients who are at high risk of the disease. The long-term goal of KD aetiology and pathogenesis research is to prevent the disease by vaccination or other strategies.

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Published online 26 March 2008

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Acknowledgements
Research in the laboratory of A.H.R. is supported by National Institutes of Health (grant HL63771) and The Kawasaki Disease Foundation of the Children’s Memorial Hospital. Research in the laboratory of S.C.B. is supported by the Research Funding Committee of Loyola University Stritch School of Medicine (grant LU10970).

FURTHER INFORMATION
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PERSPECTIVES

OPINION

Making a difference: 30 years of TDR

Robert G. Ridley and Elaine R. Fletcher

Abstract | When the UNICEF/UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR) was established in the mid-1970s, it represented an innovative institutional formula in terms of its structure and the manner in which scientists were convened from both developed and developing countries to address some of the world’s most neglected parasitic diseases. A review of TDR’s historical record sheds light not only on some important milestones in tropical disease research, but also on how future challenges could be approached and hopefully surmounted.

Institutional organization
To respond to such needs, the founders of TDR devised a set of novel institutional formulae that were inspired by some of the new public policy forums that were taking shape at the time in other arenas, particularly the Consultative Group for International Agricultural Research. Distinctive features of TDR included sponsorship by multiple UN institutions. Although TDR was initially sponsored by the World Health Organization (WHO), the United Nations Development Programme was also involved from the beginning and joined formally as a co-sponsor in 1976, followed by the World Bank in 1977 and the United Nations Children’s Fund (UNICEF) in 2003. Another innovation was the equal representation of donor and recipient governments on TDR’s governing body, the Joint Coordinating Board (JCB), which was created in 1978 (REF 5). Meanwhile, the Scientific and Technical Advisory Committee (STAC), an independent technical-oversight body, enlisted leading scientists not only from global centres of expertise but also from disease-endemic regions and industry. This kind of cooperation bestowed research credibility and helped make TDR a forerunner of the public–private partnerships (PPPs) that would emerge around the turn of the millennium6–8.

“This kind of cooperation... made TDR a forerunner of the public–private partnerships (PPPs) that would emerge around the turn of the millennium.”