The human leucocyte differentiation antigens (HLDA) workshops: the evolving role of antibodies in research, diagnosis and therapy

Heddy ZOLA*, Bernadette SWART

Leucocyte Biology Laboratory, Child Health Research Institute, 72 King William Road, North Adelaide 5006, Australia

ABSTRACT

The 8th International Workshop on Human Leucocyte Differentiation Antigens (chaired by Zola H and managed by Swart B) was run over a 4-year period and culminated in a conference in December 2004. Here we review the achievements of the HLDA Workshops and provide links to information on CD molecules and antibodies against them, including the 93 new CDs assigned in the 8th Workshop. We consider what remains to be achieved (including an estimate of the number of leucocyte surface molecules still to be discovered), and how the field can best move forward.

Keywords: leucocyte differentiation antigens, CD molecules, cell markers.

INTRODUCTION

Leucocyte cell surface molecules mediate many of the interactions between the immune system and antigens, between components of the immune system, and between the immune system and other physiological systems, such as the nervous system. Studies of immunity and diseases of the immune system have been greatly facilitated over the last 20 years by the HLDA Workshops [1-7] which have used antibodies to characterise many of the molecules involved in immunological processes, and have provided a nomenclature system, the CD system, which is used universally. The recent completion of the latest HLDA Workshop [8], the 8th, provides an opportunity to reflect on what has been achieved, what remains to be achieved, and how best to progress the field in the current state of biology.

WHAT HAS BEEN ACHIEVED

It may be difficult for the young immunologist to imagine the chaos that existed in the early days of the application of monoclonal antibodies to immunology. Many laboratories were immunising mice with leucocytes, doing fusions and cloning out hybridomas. The hybridomas made antibodies that reacted with leucocytes, but the identities of the molecular target were generally unknown. The reactivity spectrum of the antibody could be described by staining multiple different cell types, and in some but not all cases a molecular weight value could be obtained by immunoprecipitation or Western blotting.

An example will illustrate the inadequacy of this information. Several laboratories made antibodies against a protein of 24-26 kDa, expressed on platelets and non-T, non-B acute lymphoblastic leukaemia cells. Different laboratories reported different reactivities with other cells, including B cells, monocytes and activated T cells. On reading the individual papers it was far from obvious that the antibodies react with the same molecule. A major reason was that the perceived reaction spectrum depended on the questions asked. Arriving at a conclusion about identity of the antigen required special studies where the antibodies were compared side by side [9]. This example (the molecule now known as CD9, detected initially by antibodies such as BA2, FMC8, J2, DuALL1, SJ9A-4) illustrates a difficulty that was seen in many other instances.

The first HLDA Workshop, organised by Laurence BOUMSELL and Alain BERNARD [1] provided a solution to these issues by organising multi-laboratory, blind, comparative analyses of antibodies. Statistical analysis of data from several laboratories covering a number of cell types revealed “clusters of differentiation” (named for the statistical procedure of cluster analysis, and the focus on leucocyte differentiation), and immunochemical data provided supporting data in some cases. Antibodies thought to be detecting the same molecule, and the molecule itself, were given a “CD” designation. It would be difficult to exagger-

*Correspondence: Heddy ZOLA.
E-mail:heddy.zola@adelaide.edu.au

ate the importance of this process at the time – it brought order to a chaotic field.

The HLDA Workshops proceeded on a roughly 4-year cycle till the recent 8th Workshop, and each Workshop classified and named new CD antibodies and molecules (Tab. 1). During this time the field was changing, because of technical advances including particularly improvements and wider availability of flow cytometry and the development of gene cloning. In the early HLDA Workshops antibodies were made against whole cells, usually mixtures of cells, and the antibody was then used to discover the antigen. Before the wide availability of monoclonal antibodies the leucocyte molecules that were well known and characterised consisted of not much more than MHC Class I and Class II. The majority of the widely-used markers of the immune system, including CD3, CD4, CD8, CD19, CD20 and CD45 were discovered using antibodies made "blind". Even the early cloning of genes for leucocyte proteins depended first on having the antibody [10].

In contrast, all but 3-5 of the 93 CD designations allocated in the 8th HLDA Workshop were molecules which had been characterised first by identifying and expressing a gene. The antibodies were made subsequent to the discovery of the gene and expression of the protein. The role of antibodies and of HLDA has therefore changed irrevocably, and that is a good point at which to summarise what has been achieved.

First, HLDA established a nomenclature and validation process that achieved order and confidence in the use of Workshop-classified antibodies. Researchers (and especially clinical diagnostic immunologists) wanting to buy an antibody against a particular leucocyte cell surface molecule are well advised to buy one that has been validated by the HLDA Workshops. There are numerous examples of antibodies submitted as being against a particular CD turning out to be wrongly assigned by the submitters, and an antibody should not be referred to as being CD## unless this has been validated by the HLDA Workshops. A list of Workshop-validated clones may be found at www.HLDA8.org (point to the CD number in the left hand column, click and the list will appear). The same website contains a list of the newly-assigned CDs from HLDA8. A summary of the Workshop conclusions may be found in [8].

Tab. 1 Summary of the HLDA Workshops I-VIII, 1982-2004

Workshop		CDs assigned	Number of CDs assigned
I	Paris 1982 [1]	CD1-CDw15	15
II	Boston 1984 [2]	CD16-CDw26	11
III	Oxford 1987 [3]	CD27-CD45	19
IV	Vienna 1989 [4]	CD46-CDw78	33
V	Boston 1993 [5]	CD79-CDw109	31
VI	Kobe 1996 [6]	CD110-CD166	55
VII	Harrogate 2000 [7]	CD167-247	81
VIII	Adelaide 2004 [8]	CD248-339	93

Second, HLDA has provided a major forum for discussion and experimentation in the field of leucocyte molecules, and fostered a collaborative spirit of exchange of reagents and information. Interestingly, this collaborative "public good" spirit was supported by the major reagent companies, several of whom have sponsored the Workshops generously and participated actively.

Third, many of our major research, diagnostic and now therapeutic targets and antibodies derive from the HLDA Workshops – CD3 and CD20 provide important examples.

WHAT REMAINS TO BE ACHIEVED

To challenge the widely-expressed view that HLDA, after the 6th or 7th Workshop, had almost completed the catalogue of leucocyte surface molecules, we estimated that there might be more than 4000 different leucocyte membrane molecules [11-13]. The estimate is updated below:

If T cells are activated in the presence of radioactive amino acids, 2000 distinct spots can be found on 2-dimensional gel electrophoresis [14]. This is a minimum estimate because of sensitivity issues and the fact that not all molecules will be freshly synthesised in the course of the *in vitro* activation. Lefkovitz *et al* [15] in a similar study arrived at an estimate of 4000 proteins. A subsequent study from the same laboratory [16] identified 5000 RNA transcripts in a lymphocyte activation experiment. Hashimoto *et al* [17] used SAGE (serial analysis of gene expression) to look at sequences expressed by different leucocytes, and concluded that there were 27,000 tags representing unique genes expressed in leucocytes of different types. Any individual cell type expressed 7000 to 16,000 distinct genes. These estimates vary because of the different approaches used and differences in sensitivity. They yield numbers of expressed genes or proteins, not restricted to the membrane. Analysis of the human genome suggests that 20% of genes code for membrane proteins [18]. Combining this figure with the estimates of proteins or RNA species suggests a range from 400 (activated T cells only) to 5,000 membrane proteins. Only a fraction of membrane molecules are in the external plasma membrane of the cell, and we have no estimate for the size of this fraction, other than to note that molecules which clearly function primarily on the membrane of intracellular organelles are increasingly found also on the plasma membrane. We need to reduce the estimate to allow for membrane proteins not found on the extracellular membrane, but we do not know

by what factor. On the other hand, each mRNA species can lead to multiple proteins through post-transcriptional modifications, so we need to increase the number, by an unknown factor. If, in our ignorance, we allow these factors to cancel each other, we are left with an estimate of 400 (activated T cells only) to 5000. Among the known CD molecules, 70/400 are designated as expressed on T cells, suggesting that the lower estimate should be increased to about 2,400.

The estimate of 2,400-5000 leucocyte cell surface molecules is subject to a number of caveats, but is used only to conclude that the catalogue is not yet close to complete. This conclusion is supported strongly by two sets of data. The first is the increasing numbers of CDs allocated at succeeding HLDA Workshops (see Tab. 1). If we were nearly finished with the catalogue, we would expect to see diminishing returns. The second is the large numbers of new proteins that can be found in proteomic analyses of cell membrane extracts [19].

Discussion thus far has focussed on membrane-expressed proteins, but intracellular molecules can also serve as markers of lineage and differentiation stage, and can also be used as diagnostic markers [20].

To summarise what remains to be done, there are many more molecules on the surface of leucocytes that have yet to be discovered, or if they have been discovered antibodies against them are not yet available or have not been subjected to Workshop evaluation. In addition to these surface molecules, there are potentially many intracellular molecules that can also serve as diagnostic or research markers of differentiation and cell lineage.

THE WAY FORWARD

The HLDA Council reviewed the objectives and operation of HLDA at the end of the 8th HLDA Workshop. The consequences of that review may be seen in the paper summarizing HLDA8 [8] and on the HLDA website (www.hlda8.org, see the *HLDA to HCDM* link). Key changes are to broaden the scope to include molecules that are of value in studies of cellular immunity but are either not primarily on the cell surface or not primarily on leucocytes, to focus more on validation of antibody reagents and less on assigning CD names (molecules will receive CD designations if the Council feels this will assist scientific communication, but CD names will not necessarily be assigned to well characterised molecules with unambiguous names). These changes will take effect after HLDA8, so HLDA8 assigned CD designations to molecules such as TNF superfamily members and Toll-like receptors, which arguably do not need additional names.

To reflect these changes HLDA will take on a new name (HCDM, for Human Cell Differentiation Molecules). However for continuity and ease of access (eg to the web site) the name HLDA will also be retained. HCDM will move from a 4-year cycle to an approximately annual cycle, but each cycle will be focussed, rather than encompassing all aspects of human cell differentiation molecules. Details of HCDM round 1 can be found at www.hlda8.org.

Progress in the field of leucocyte molecules is no longer focussed largely by the HLDA Workshops, as it used to be. Most molecule discovery in the last few years has come from gene cloning studies, and in our view most molecule discovery in the next few years will come through proteomic studies [13, 19]. Nevertheless, neither cDNA nor proteomic technology provide a substitute for antibody-based methods for research, diagnosis and therapy. To immunologists accustomed to flow cytometry and immunhistochemistry data, tissue distribution data based on expression arrays look very imprecise and incomplete. Such data are useful only until an antibody is available. DNA-based techniques such as PCR provide advantages of sensitivity in some aspects of diagnosis, but antibody-based methods provide opportunities for multiparameter and multiplex analyses which add resolving power to differential diagnosis. One example of the value of multiparameter analysis in pathophysiological studies which may lead to new diagnostic tests is the resolution of memory T cells into effector memory and central memory cells [21]. An example of the potential value of multiplex assays is the application of cytometric bead arrays to measure multiple cytokines in the diagnosis of sepsis in neonatal infants [22]. As recently pointed out by Ulloa and Tracey [23] sepsis is a complex and diverse set of conditions, and understanding the factors involved in each patient could lead to optimised therapy. The potential for antibody-based therapy is being realised rapidly [24, 25] after a long lag time when OKT3 was the only broadly successful therapeutic antibody.

In conclusion, antibodies retain a major role as research reagents and an increasingly powerful role as diagnostic reagents. Antibodies as therapeutic agents have emerged from a long "dark age" and have become the fastest-growing area of biological therapeutics. HLDA has been central to these developments in the context of leucocyte targets, and, by adapting to the changing environment, can maintain its central role. The approach taken by HLDA may well be taken up to advantage by other fields of biology and medicine. And finally, we have identified a major window of opportunity for discovery of many more leucocyte surface molecules, among which we would expect to find new targets for diagnosis and therapy.

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