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Research paper

The HLDA8 blind panel: Findings and conclusions

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Abstract

There were over 600 antibodies submitted to HLDA8, with many of unknown specificity. Of these, 101 antibodies were selected for a blind panel study that also included 5 negative controls and 27 positive controls of known CD specificity making a total of 133 antibodies in the final panel. Of the 101 unknowns, 31 antibodies were identified during the course of this blind panel study as being specific for known molecules and included some specific for MHC class II antigens, CD45 isoforms and the Dombrock antigen. Several antibody pairs among those in the blind panel were found to have very similar staining patterns and were therefore compared by immunohistochemical and/or Western blot analyses for identity.

1. Introduction

Historically, each of the HLDA workshops has received a large number of antibodies that are directed against unknown molecules. The original CD nomenclature ("Cluster of Differentiation") was based on the concept of detecting groups of antibodies that showed similar patterns of reactivity (exhi-

Abbreviations: CD, Cluster of Differentiation; HLDA8; 8th International Workshop on Human Leucocyte Differentiation Antigens.

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bited by immunofluorescence) when analysed against a large variety of different cell types. Such analyses have always been conducted in multiple laboratories using blinded samples. This has ensured that any biased results associated with either staining technique or expected reactivity have been avoided. Results obtained from any laboratories that scored poorly on tests using control antibodies were excluded from further analysis.

As the HLDA proceeded through the 3rd to the 6th Workshops it became increasingly useful to include known CD antibodies as controls, so that new antibodies against these CDs could be identified by their similarity to the known antibodies. On the other hand, the increasing power of molecular techniques meant that a greater number of antibodies were submitted with known specificity, in many cases because they had been made against recombinant protein or transfected cells.

The results obtained from Blind Panel studies undertaken as a part of the 6th HLDA Workshop resulted in the value of this method being questioned. The consensus opinion reached was that the use of this approach had limited value and so no Blind Panel study was undertaken as part of the 7th HLDA Workshop. For the 8th Workshop it was decided to have a limited Blind Panel, focusing on antibodies that could not be assigned to a known molecule on the basis of information supplied by the submitting laboratory or as a result of preliminary examinations undertaken by the Organising Laboratory or Section Chairs.

2. Materials and methods

2.1. Composition of the HLDA8 blind panel study

For the 8th Workshop, it was decided to conduct a limited blind panel study that comprised antibodies submitted to HLDA8, but excluding antibodies where the specificity had been identified by the submitting laboratory or ascertained by preliminary investigations undertaken by the organizing laboratory or section chairs. This process reduced the number of candidate antibodies from over 600, to a panel of 101 unknown antibodies, which was then supplemented by the inclusion of 5 negative controls and 27 antibodies of known specificity to give the final Blind Panel of 133 antibodies. These antibodies were submitted by laboratories from Australia, Canada, China, Czech Republic, Denmark, Estonia, France, Italy, Japan, Netherlands, Russia, Thailand, United Kingdom and USA, with the analyses being designed and conducted by collaborating groups based in Australia, Czech Republic, Turkey, United Kingdom and USA as illustrated in Fig. 1.

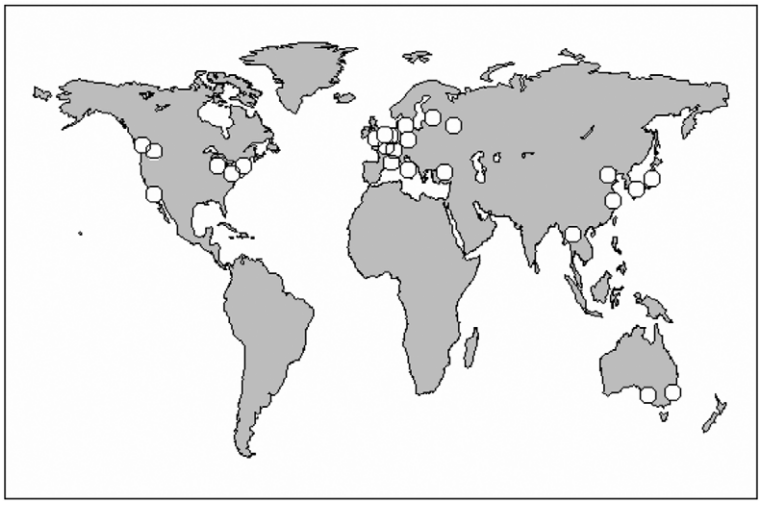

Fig. 1. This work required the close collaboration of a number of laboratories around the world. Each dot locates a city where one or in some cases multiple laboratories were involved by way of the production and/or analysis of antibodies for this blind panel study.

Each antibody in the Blind Panel was evaluated against a panel of cell populations (Table 1), with a large univariate sample of fluorescence measurements being collected for each population of cells examined after staining with antibody. Where antibodies were not found to stain lymphocytes, subset analyses were not performed.

The Blind Panel studies were not designed to identify new antibodies against known molecules, so no attempt was made to include controls for every known CD. The reference antibodies were included for the calibration of the semi-automated process of detecting the groups of antibodies with similar reactivity patterns. In order to calibrate the algorithm used for the detection of antibodies with the same reactivity, the controls were selected to include multiple antibodies against a small number of different specificities. Five antibodies were omitted from the control panel as they were found to give staining profiles that were not consistent with their supposed specificity. The final group of control antibodies used for these studies included three negative control antibodies, eight monoclonals that recognised CD3, four that were specific for CD14, three that bound to CD15, four directed against CD19 and six anti-CD45RA monoclonal antibodies. There were also a number of diluted monoclonal antibody preparations included to test the ability of analysts to recognize antibody identity and these included two dilutions of anti-CD3, 4 dilutions of anti-CD14, two dilutions of anti-CD19

Table 1
Target cells stained during blind panel analysis

Cell line	Abbreviation	Cell line	Abbreviation
Resting Peripheral Blood Lymphocytes[a]	REST.PBL.BD	Whole Blood Lymphocytes[b]	WBL
Activated Peripheral Blood Lymphocytes[a]	ACT.PBL	Whole Blood Monocytes[b]	WBM
Cell line 697[a]	697	Whole Blood Granulocytes	WBG
Cell line A431[a]	A431	Whole Blood CD4+[b]	WB.4.45.4
Cell line HELA[a]	HELA	Whole Blood CD4+CD45RO+[b]	WB.4.45.RO
Cell line HL60[a]	HL60	Whole Blood CD4+CD45RO−[b]	WB.4.45.RO−
Cell line HUT78[a]	HUT.78	Whole Blood CD8+[b]	WB.8.45.8
Cell line JURKAT[a]	JURKAT.BD	Whole Blood CD8+CD45RO+[b]	WB.8.45.8B
Cell line RAJI[a]	RAJI.BD	Whole Blood CD8+CD45RO−[b]	WB.8.45.8A
Cell line U937[a]	U937	Whole Blood CD19+[b]	WB.19.56.19
Cell line DHL-4[c]	DHL4	Whole Blood CD56+[b]	WB.19.56.56
Cell line OPM1[c]	OPM1		
Cell line JJN3[c]	JJN3	Whole Blood Lymphocytes[d]	FMC.L
Cell line KM518[c]	KM518	Whole Blood Monocytes[d]	FMC.M
Cell line NALM6[c]	NALM6	Whole Blood Granulocytes[d]	FMC.G
Whole Blood Lymphocytes[c]	PBL.L	Whole Blood CD19+[d]	FMC.19
Whole Blood Monocytes[e]	PBL.M	Whole Blood CD4+[d]	FMC.4
Whole Blood Granulocytes[e]	PBL.G	Cord Blood Lymphocytes[f]	CBL
Whole Blood CD19+[e]	WB.4.19.19	Cord Blood Monocytes[f]	CBM
Whole Blood CD4+[e]	WB.4.19.4	Cord Blood Granulocytes[f]	CBG
Activated Peripheral Blood Lymphocytes CD4+[e]	PBL.4	Cord Blood CD4+[f]	CB.4.45.4
Activated Peripheral Blood Lymphocytes CD19+[e]	PBL.19	Cord Blood CD4+CD45RO+[f]	CB.4.45.RO
Activated Peripheral Blood Lymphocytes CD56+[e]	PBL.56	Cord Blood CD4+CD45RO−[f]	CB.4.45.RO−
Activated Peripheral Blood Lymphocytes CD4−CD56−[e]	PBL.456	Cord Blood CD19+[f]	CB.19.56.19C
Cell line JURKAT[e]	JURKAT	Cord Blood CD56+[f]	CB.19.56.56C
Cell line THP-1[e]	THP1	Lymph Node[f]	LN
Cell line RAMOS[e]	RAMOS		

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Table 2
Antibodies identified during the course of blind panel analysis

Antibody no	Antigen identity	Antibody identities
80364	CAM36A	CD14
80370	H18A	
80253	B-A8	
80636	MY4	
80640	MI02	
80254	B-H8	CD15
80371	RACT48A	CD18
80269	B-D3	CD19
80418	HI313	CD44
80204	ZCH-5E2	CD45
80260	B-A11	
80261	B-B3	
80262	B-C15	CD45RA
80365	DH16A	CD45RB
80101	MIMA 52	Dombrock antigen
80102	MIMA 53	(CD297)
80188	5F1	FMLP receptor
80278	E63-761	MHC Class II
80341	CR3/43	
80395	HI220	
80397	HI301	
80398	HI305	
80399	HI306	
80400	HI307	
80443	HI302	
80199	P-3E10	Na/K ATPase, beta3 subunit (CD298)
80533	N3.12.4	Nectin 3 (CDw113)
80534	N3.82.5	
80535	N3.81.6	
80426	0	PAI 1
80428	C12	

and one dilution of the anti-CD45RA monoclonal antibody preparations. These dilutions were however excluded from the statistical analyses. The statistical approach used for these analyses was designed specifically for this work (Salganik et al., in press) and is described fully in the accompanying paper (Salganik et al., 2005—this issue).

All laboratories participating in the collection of Blind Panel data, with the exception of the Organising Laboratory, were blind to the identity of the controls. In the Organising Laboratory the person running the tests and flow cytometric analysis was also blind to the identity of the controls. The person responsible for the statistical analyses (MS) was given details of the specificity of the antibodies that made up the group of known controls and the identity of negative controls. This information was used in the statistical analyses.

Six laboratories performed flow cytometric analysis and of these, two also performed Western blot analyses on selected antibodies to confirm or negate suspected identities and one additional laboratory performed immunohistochemical analyses. Table 1 lists the laboratories involved and studies performed.

3. Results

During the early stages of the blind panel studies 24 antibodies of the 101 "unknowns" were identified as being specific for known molecules and one "dim" staining antibody was removed from the analysis, leaving 76 antibodies of unknown specificity for analysis. In some cases, this identification occurred during the initial examination of expression profiles generated from the staining of resting and activated blood cells, when similarities to the expected patterns of known CD molecules were noticed. Suspected specificities were confirmed by Western blot analysis. In the case of antibodies against the Dombrock antigen, identification came from the submitting laboratory during the course of the studies. The 24

Table 3
Outcome for antibody pairs showing high (>0.85) correlation

Antibody number	Clone name	Findings	Conclusion
80196 80197	BC213 BK68	Western blots were negative	Inconclusive
80291 80292	LT-ND5 LT-ND4	Western blots were negative, both are IgM antibodies	Inconclusive
80598 80600 80601	MEM-114, MEM-187 MEM-207	Submitter's data show differences	Non-identical
80041 80406	B-F16 HI263	Western blotting showed bands at 64 kDa	Potential cluster, merits further study
80039 80040 80405 80407	B-H14 B-E22 HI265 HI267	Did not show clear bands	Potential cluster, merits further study

antibodies identified in this way are listed in Table 2, and include 8 antibodies against MHC Class II and 5 against CD45 isoforms. The identification of the specificities of 24 antibodies at this early stage of analysis illustrates one advantage of the strategy adopted by the blind panel study. The specificities of an additional seven antibodies also in Table 2 (80253, 80254, 80269, 80371, 80398, 80636 and 80640) were not known at the time when the analyses of data presented by Salganik et al. (2005—this issue) was performed, so their identities were assumed to be unknown in the analyses described by these authors.

Analysis of the flow cytometric data for the remaining unknowns as described in the accompanying paper (Salganik et al., 2005—this issue). Resource limitation caused us to focus immunochemical analyses on 28 pairs formed by 25 antibodies of unknown specificity with very similar reactivity patterns (correlation > 0.85) (Table 3).

A summary of the detected similarities in reactivity of antibodies is presented in Fig. 2. Where a block designating a pair of antibodies is shaded grey, this indicates a strong similarity of staining patterns. The symbols "+" or "−" within a square indicate that identity was subsequently confirmed or rejected, respectively. It is clear from the data presented in Table 3 that a number of antibodies were found to react against the same antigens or at least the same cell types, but some additional studies will be required to fully identify the specificity of many of these antibodies.

While pairs of antibodies that are specific for the same molecules may possibly be found among the unknowns in the group with the lower correlations, with the limited time available for completion of Workshop studies, we focussed biochemical analysis on a small group of antibody pairs with very high correlation (>0.85) (Table 3). It is worth noting that not all pairs identified in this way were found to be specific for the same antigen. One clear example of this is the direct comparison of antibodies 80600 and 80601 which were shown to have a high coefficient of

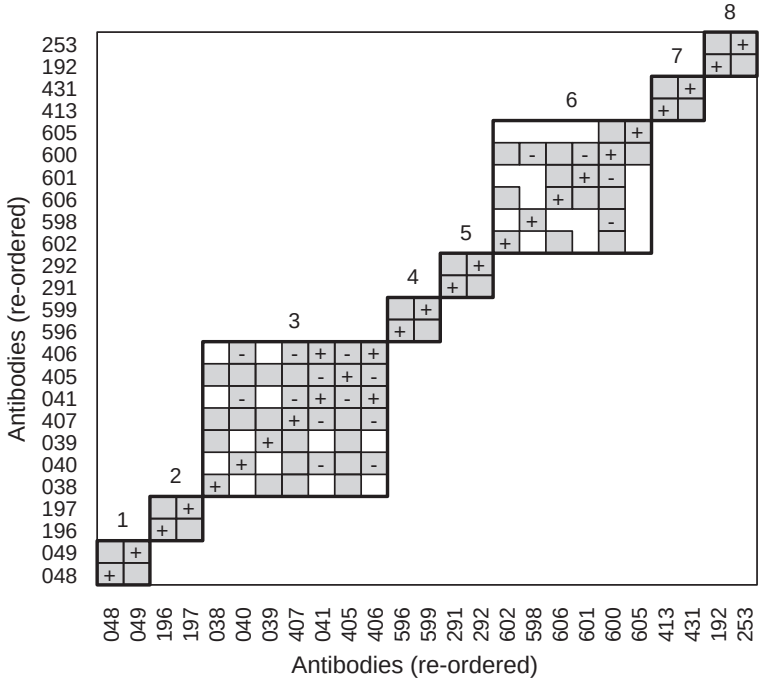

Fig. 2. This graph illustrates the similarity of specificities for antibodies that is based on the analysis of immunofluorescence and biochemical data. The grey shading indicates pairs of antibodies with similar patterns of immunofluorescence. The symbols within the grey squares mark the pairs for which identity was confirmed ("+") or rejected ("−") based on Western blot and immunohistochemical analyses. Note that the first two digits of the antibody identification numbers are not shown here for the sake of clarity. The antibodies 80600 and 80601 provide one example of a pair that was subsequently found not to be identical in their reactivity.

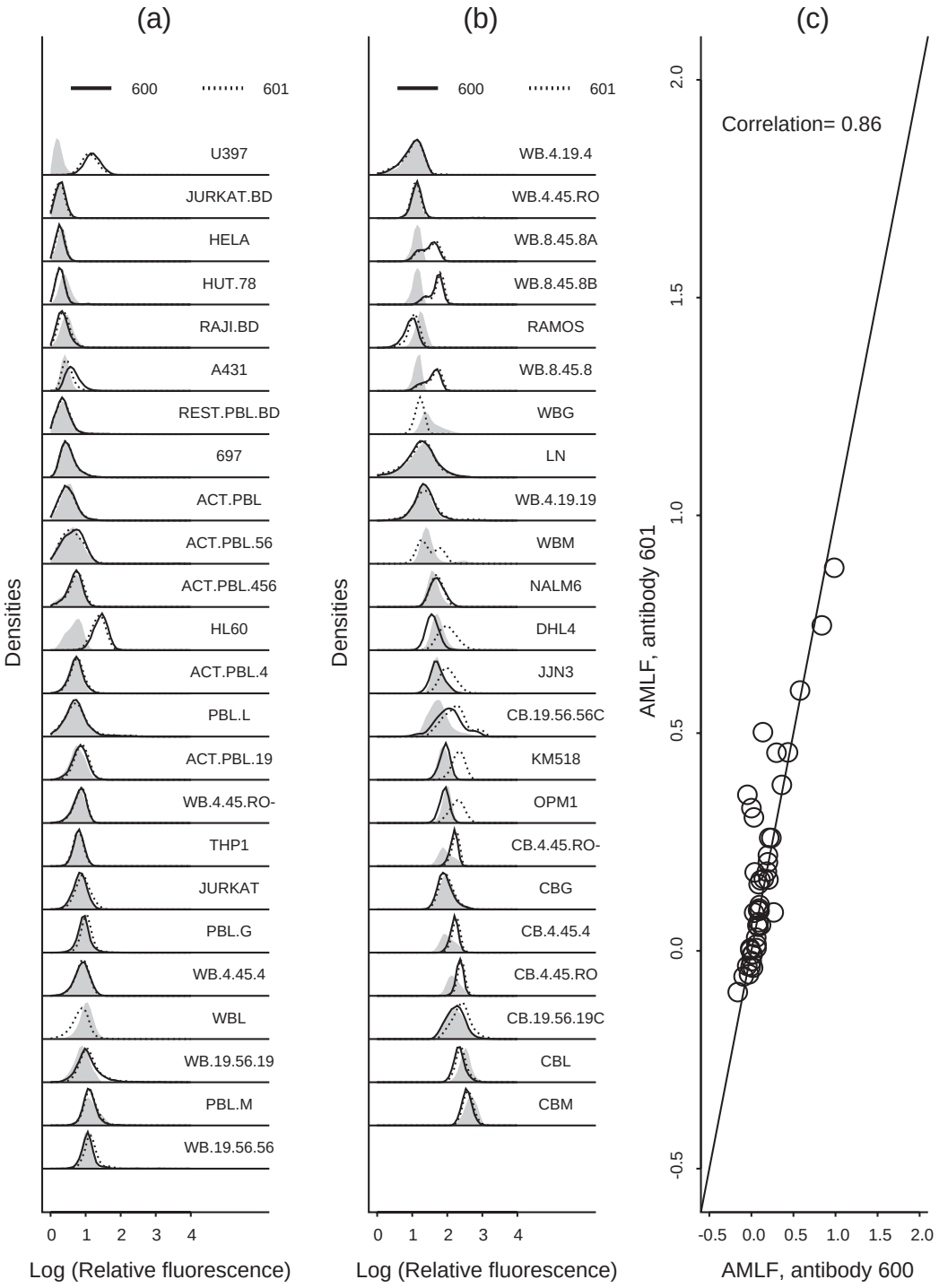

Fig. 3. (a,b) Density estimates of log fluorescence intensity distributions for negative control—stained cell populations (grey polygons) and cell populations stained by antibodies (solid line, antibody 80600 and dashed lines, antibody 80601). The density curves are scaled so that the maximum value for each of the cell populations is equal to one. (c) A comparison of the baseline adjusted values of mean log fluorescence (AMLF). Each symbol represents the value for a particular cell population stained by antibodies 80600 and 80601.

correlation (>0.85) (Fig. 3), yet differences were apparent in both Western blot and Immunohistochemical analyses. The Western blot analysis shows the presence of an additional band of high relative molecular weight for antibody 80601 when compared with antibody 80600 (Fig. 4). Similarly antibody 80601 was found to exhibit a far more intense and widespread pattern of staining than 80600 when the two were compared on sections of human umbilical cord (Fig. 5a and b). Both these tests provide compelling evidence to suggest that the two antibodies are not recognising the same epitope in spite of their high correlation, and may simply indicate that both antigens have very similar patterns of expression on the cell types tested.

In the limited time available for completion of the workshop studies, we focussed the biochemical analyses on a small group of antibodies, which exhibited very similar reactivity patterns and sufficient quantities were available for the investigations required. The results of this work are summarised in Fig. 2 and Table 3. Based on the data currently available, it is anticipated that antibodies 80041, 80406 and antibodies 80039, 80040, 80405, 80407 may exhibit identical reactivity patterns.

During the process of analysing flow cytometry data described by Salganik et al. (2005—this issue), the specificity of antibody 80636 was not known but a striking similarity with the reactivity patterns of this and antibodies known to be specifically directed against CD14 was noted. Biochemical analysis has now confirmed that this antibody binds to the CD14 antigen (Table 2).

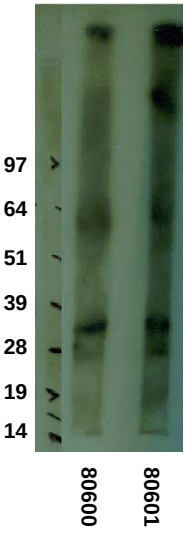

Fig. 4. Western blot analysis of antibodies 80600 and 80601 shows some differences with the detection of a high molecular weight band seen with antibody 80601 but not with antibody 80600.

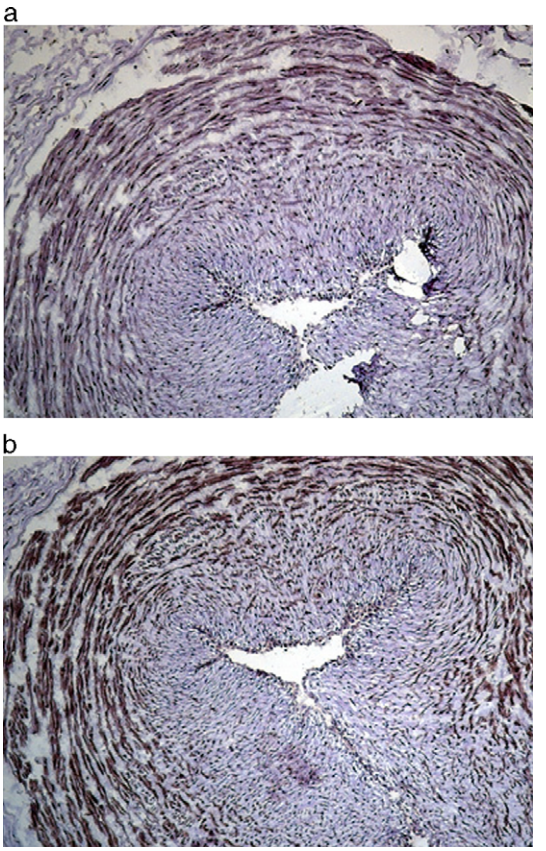

Fig. 5. Immunohistochemical staining of human umbilical cord sectioned through the umbilical artery, showed some differences in the reactivity patterns for antibodies 80600 (a) and 80601 (b) with a greater number of cells being more intensely stained with antibody 80601.

4. Discussion

Large numbers of antibodies are submitted to HLDA Workshops. Some are submitted as being against a known molecule and can be confirmed by reactivity against transfected cells or recombinant protein. Others are submitted as unknowns, and are often submitted with minimal information about their

specificity. Preliminary analysis by the participating laboratories often leads to tentative identification of antibodies as being against a known molecule, and this can be confirmed using specifically transfected cells or recombinant protein, by immunochemistry or by competitive studies with known antibodies. The use of such approaches, as well as the elimination of weakly staining antibodies, enabled us to reduce the list of over 600 antibodies initially submitted to the final 101 that, together with the set of control antibodies selected, constituted the "blind panel" study described here.

Identification of specificity before antibodies were directed to the Blind Panel was not systematic, but rather depended on the observations and guesses of the investigators. Consequently, 31 antibodies of the 101 originally assigned to the Blind Panel were identified during the course of the Blind Panel studies as being directed against known molecules. Of these, 24 were identified prior to statistical analysis and were therefore eliminated from the statistical analyses presented in the accompanying paper (Salganik et al., 2005—this issue). Closer examination of the submitting laboratories' data also resulted in the elimination of three pairs, which were found to be specific for different molecules in spite of having high correlation values.

Antibodies from the three remaining groups were tested by Western blotting, with data from two of the groups being inconclusive. In Western blot assays conducted with the remaining group of 6 antibodies two gave bands of similar molecular weight, while the others did not give bands. While this group clearly warrants further investigation, the fact that four of the six antibodies failed to produce bands on a Western blot illustrates the value of being able to assess the binding characteristics of such antibodies by a number of different techniques.

Clearly, this leads back to consideration of the future place of Blind Panels in the analysis of human cell differentiation molecules. Advances in protein chemistry and molecular biology mean that it is now relatively easy, given an antibody against a novel protein, to isolate the protein, determine partial protein sequence and then clone the gene. Nevertheless, when faced with hundreds of antibodies, the prospect of doing an equivalent number of immunoprecipitations, sequence analysis and cloning is a daunting and expensive task. Such an undertaking would also be made more complex by the simple fact that immunoprecipitation reaction mixtures will often need to be optimised for each antibody, and some antibodies will fail both in immunoprecipitation and Western blot analyses. Whether immunofluorescence staining profiles remain the sole or even the key source of data for the classification of antibodies or the use of microarray based assays like those described by Belov et al. (2001) may supplement or even replace them may be an issue worth consideration.

Past experience indicated that even after eliminating antibodies against known molecules by analysis with some carefully selected target cells, there was likely to a number of unidentified antibodies remaining—in the 8th workshop, there were 76 antibodies that fell into this category. That was still too large a number to handle easily by immunoprecipitation and proteomic analysis, and any panel of this size will undoubtedly contain a number of antibodies that are specific for carbohydrate determinants, which are as yet not readily amenable to identification by mass spectrometric analysis. On the other hand, the Blind Panel is a very significant investment of time by a number of laboratories, and does not provide a definitive identification of specificity.

The work undertaken as a part of the HLDA8 workshop shows that analysis by immunofluorescence is a very good way to eliminate antibodies against known antigens first, thereby reducing the number of unknowns. However, this is probably best not done in a "blind" panel.

It seems very likely that improvements in proteomic technology and related techniques that are required to handle carbohydrates will eventually make identification of antigen routine. Until this becomes reality, there is a place for a Blind Panel to identify groups of antibodies which merit further evaluation. Careful selection of target cells and improved methods of data analysis, such as those described by Salganik et al. (2005—this issue, in press) can and will make this process easier and much more efficient than it has been in the past. Our work confirms the experience of earlier workshops (e.g. Gilks and Shaw, 1995) and suggests that a detection of the similarity in the patterns of reactivity for antibodies provided by the blind panel, gives a valuable insight to the antibodies' specificity. However, analyses that are based

on a limited panel of indicative cells cannot provide definitive proof of the identity of specificity for a pair of antibodies and so the follow-up use of biochemical methods is essential.

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