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APPLICATIONS OF ENZYMATIC AMPLIFICATION STAINING IN IMMUNOPHENOTYPING HEMATOPOIETIC CELLS

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1. ABSTRACT

Immunofluorescent staining of mammalian cells has provided a reliable approach for detection of specific antigen expression *in situ.* An advantage of fluorescent markers has been their applicability to automated, high-throughput cellular analysis by flow cytometry. Flow cytometry has thus become an integral component of clinical laboratory diagnostics, particularly in the areas of immunology and hematology. One of the major drawbacks of traditional immunofluorescent staining, even with flow cytometric detection, has been the difficulty in detecting low abundance cellular antigens, some of which may have clinical and scientific significance. To address these problems, staining techniques have recently been developed to increase the sensitivity of cellular antigen detection by flow cytometry. In this review we will describe a few of these techniques and focus on enzymatic amplification staining as a means to generate a highly augmented antigen-specific signal. We will also discuss practical applications of enzymatic amplification for immunostaining of clinical specimens.

2. INTRODUCTION

Detection of antigens in tissues by specific antibody probes has been crucial for identification, classification and understanding of an array of diseases. A number of direct methods have been developed to trace antigen-bound antibody including direct conjugation of antibody with fluorescent label or direct conjugation of antibody with enzymes acting on chromogenic substrates. Indirect methods, by contrast, use tagged secondary reagents to detect antibody staining of tissues and tend to provide enhanced sensitivity of detection. Both direct and indirect methods may be applied to cells in tissue sections (immunohistochemistry) and in individual cell suspensions (flow cytometry). Chromogenic methods are routinely employed for immunohistochemistry and are not applicable to flow cytometry. In flow cytometry, fluorescence-based detection has provided several advantages including superior sensitivity, adaptability to automated signal detection, and the capacity for simultaneous staining of cells with multiple distinct fluorochromes.

Immunohistochemistry permits direct correlation of histology with specific antigen expression but permits evaluation of only small numbers of cells with relatively high levels of target antigen expression. Flow cytometry is a more sensitive antigen detection method in which multiple antigens can be analyzed simultaneously on tens of thousands of cells in a few minutes. The analytical power of flow cytometers has led to their routine incorporation into clinical laboratories for evaluation of hematologic malignancies, immunodeficiencies, hematopoietic stem cell quantitation for bone marrow transplant, analysis of cellular DNA content, the evaluation of fetal-maternal hemorrhage, and the identification of

allogeneic antibodies. Analysis by flow cytometry is still limited, though, by the type of cells that can be analyzed, the relatively large number of cells required, the need for fresh specimens, the inability to correlate directly with histology, the unavailability of certain antibodies, and the insensitivity of the analytical method.

Analytical sensitivity applies to two different variables: the detection of rare events and the detection of weakly expressed antigens. While the former has been an area of significant clinical research because of its importance for the detection of minimal residual disease, advances in the latter area have not been pursued vigorously for flow cytometry. The absence of interest in enhancing fluorescent signals is likely due to the already superior sensitivity of immunofluorescent staining and flow cytometry as compared with immunoperoxidase-based colorometric methods for the detection of cellular antigens in tissue specimens (1). Flow cytometry has also proven more sensitive than complement-dependent cytotoxicity assays for identification of allogeneic HLA antibodies in clinical laboratories (2). Below we describe methods to augment fluorescent signals using a recently adapted enzymatic amplification technique based on tyramide deposition (3,4). We also discuss the clinical applications of fluorescence signal amplification for flow cytometry and for other diagnostic modalities.

3. METHODS TO ENHANCE SENSITIVITY USING FLUORESCENCE

3.1 Optimizing the signal to noise ratio

The sensitivity of any assay system is dependent upon signal to noise ratio. In flow cytometry the signal is dependent upon the inherent brightness of the fluorochrome signal, and the noise derives from cellular autofluorescence and non-specific adherence. With standard direct immunofluorescent staining and flow cytometry, it is estimated that several thousand copies of surface antigen are needed to discriminate true positive cells from background autofluorescence (5). Watson and Walport estimated a detection limit by flow cytometry with directly conjugated antibodies that was on the order of 1000 target antigens per cell (6). Therefore the ability to detect antigen at lower levels may be lost using standard direct immunostaining techniques.

Cellular autofluorescence is an intrinsic property of cells due to the presence of intracellular flavin compounds and NADH (7). These ubiquitous biomolecules excite over a wide range of wavelengths, the most troublesome of which is 488nm, the excitation wavelength produced by the argon lasers in most flow cytometers (7). The peak emission after 488nm excitation is in the green region of the spectrum, leading to interference with the detection of FITC emission (5).

Improving the signal to noise ratio by reducing background autofluorescence has been the focus of a number of studies (8-10). Differential excitation of fluorochrome and cellular autofluorescence can be achieved using a dual laser approach, allowing for correction of background noise and amplification of the specific signal (9). A novel phase-sensitive detection system is capable of resolving two different fluorescent compounds with similar emission spectra based on their fluorescent half-life (10). These methodologies require specific instrumentation and are not practical for most standard flow cytometry laboratories. The addition of trypan blue or methylene blue to fixed, permeabilized cells has been used to quench background autofluorescence (8). Although a five-fold increase in the signal to noise ratio can be attained using this simple technique, careful titration of the dye concentration is necessary to accomodate variability in cellular susceptibility to autofluorescence quenching and to avoid quenching of specific signal (8).

The signal to noise ratio is also determined by signal intensity, which in turn is a function of the inherent "brightness" of the fluorochrome and the number of fluorochrome molecules conjugated to the antibody. For any antibody, the signal to noise ratio can vary 4- to 6-fold depending on which fluorochrome is conjugated. The relative brightness of fluorochromes on dual laser Becton Dickinson instruments is (from brightest to dimmest): allophycocyanin, phycoerythrin, phycoerythrin-Cy5, fluorescein isothyacyanate, and peridinin chlorophyl protein. The number of fluorochromes conjugated to an antibody is expressed as the F/P ratio. A higher F/P ratio indicates more fluorochrome is conjugated to the antibody resulting in a stronger fluorescent signal. The physical properties of both fluorochrome and antibody limit the number of fluorochrome molecules that can be conjugated to a maximum of 10 fluorescent molecules per antibody. These physical properties determine the limits of antigen detection on cells by flow cytometry.

3.2. Indirect immunofluorescence

To enhance the sensitivity of flow cytometry, attempts have focused on strengthening the fluorescent signal. The simplest strategy has been to use indirect immunofluorescent staining in which an additional secondary, tertiary or quarternary labelled component amplifies the fluorescent signal. Zola *et. al.* reported that a three-layered technique, consisting of monoclonal primary antibody, biotinylated polyclonal secondary antibody, and phycoerythrin-streptavidin tertiary layer, could detect less than 100 molecules per cell, a ten-fold improvement over direct immunostaing methods (11). This technique identified on resting lymphocytes surface interleukin 2 receptor beta chain (CD122), which could not be identified by other standard methods (11). Laszlo and Dickler using a similar three-tiered method detected surface antigens at a lower limit of an estimated 200 copies per cell (12). Cohen *et. al.* used a sandwich of biotinylated primary antibody followed by phycoerythrin-streptavidin followed by biotinylated goat anti-streptavidin antibody and another round of staining with phycoerythrin-streptavidin to generate a highly sensitive immunofluorescent probing system with a reported antigen detection limit of 50 copies per cell (13).

There are several instances in which expression of surface proteins can be demonstrated by biological activity but cannot be detected by indirect

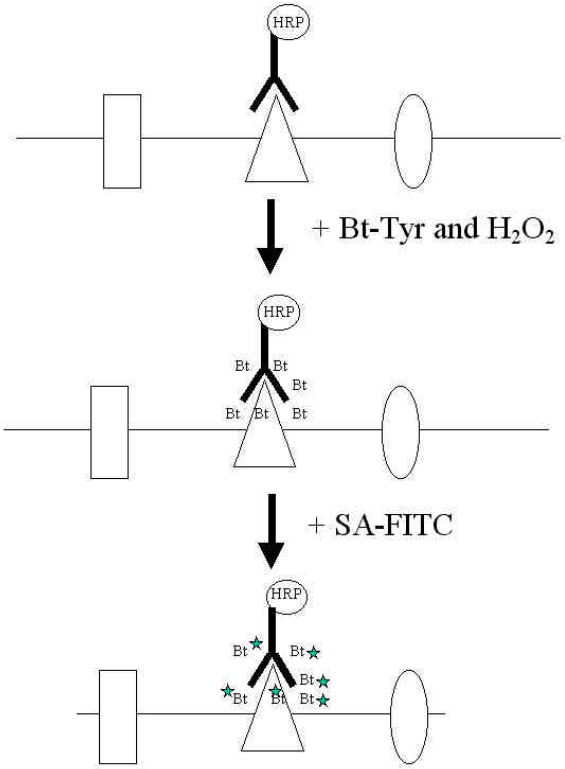

Figure 1. Diagram of enzymatic amplification. In a typical enzymatic amplification reaction, HRP-conjugated antibody binds to a specific cell-bound antigen. The addition of biotinylated tyramide (Bt-Tyr) and hydrogen peroxide leads to the activation of highly reactive tyramide radicals that react with exposed phenolic groups leading to numerous biotinylated adducts (Bt). Detection is accomplished by the addition of streptavidin-FITC (SA-FITC) leading to the abundant tagging of the cell by many fluorescein compounds (∠).

immunofluorescent immunostaining (13). For example we observed Fas ligand activity on cytotoxic T cell clones by a highly sensitive bioassay but could not detect expression by direct or enhanced indirect immunostaining (3). Similarly, others have documented cell surface cytokine activity in the absence of molecules detectable by conventional staining techniques (14-16). Another weakness of indirect immunofluorescent staining is difficulty with multicolor analysis due to cross-reactivity of secondary antibodies. Therefore attempts to amplify signals based on this approach are generally limited to two colors.

3.3. Liposome-conjugated Antibodies

In an attempt to link more fluorescent molecules to antibodies to increase the F/P ratio, several groups have used liposomes as fluorescent carriers (17-21). Functioning as vesicles loaded with fluorochrome, liposomes can be conjugated to antibodies to generate a highly sensitive direct tag. As opposed to the typical F/P ratio of 2-5 on direct fluorochrome conjugated antibodies, F/P ratios of 100-1000 may be achieved with liposome conjugated antibodies, providing proportional enhancement of fluorescent signal with no increase in background fluorescence. This method was first utilized by Truneh *et. al.* (17,21) and more recently by Scheffold et. al., who co-loaded liposomes with magnetic particles to facilitate cell separation (18,19). The latter group was able to detect 200-300 target antigens per cell, a sensitivity similar to that obtainable with indirect immunofluorescence. The liposomal technique in their hands permitted clear detection on activated T cells of low level surface levels of IFNγ and IL-10 , previously only inferred from bioassays. Klein *et. al.* used liposome conjugated antibodies for analysis and isolation of peripheral blood B cells weakly expressing CD27, a marker of post-germinal center memory B cells that have previously undergone antigen selection (20). A major drawback of using liposome-antibody conjugates is that the method is unsuitable for intracellular staining due to the bulk of the liposome conjugate. In addition, one must avoid exposure of liposome-antibody conjugates to detergents, which will disrupt the fluorochrome-laden micelles.

3.4. Enzymatic amplification staining

To detect low abundance surface and intracellular antigens, we and others have employed enzymatic amplification of the fluorescent signal. This amplification uses horseradish peroxidase-conjugated primary antibodies in conjunction with fluorochrome-labeled tyramide compounds (Figure 1). When oxidized by horseradish peroxidase, the phenolic tyramide generates free radical intermediates and undergoes concentration dependent dimerization (22-24). At low tyramide concentrations, the probability of homo-dimerization diminishes and the probability increases of hetero-conjugation to electron-rich regions of proteins, such as tyrosine side chains, near the site of the peroxidase reaction. Thus a large amount of labelled tyramide can be introduced in proximity to the antibody binding site. Because the reactive tyramide intermediates are extremely short lived, the probability is minimal of tyramide conjugating with proteins distant from the antibody binding site or on bystander cells.

This detection system was first developed by Bobrow in 1989 for solid phase immunoassays (22,23). The method has been variably termed catalyzed reporter deposition (CARD), tyramide signal amplification (TSA) or enzymatic amplification staining (EAS). Shortly after its initial description the method was adapted for immunohistochemistry (23,25-28), *in situ* hybridization (24,29) immunoelectron microscopy (30,31), and more recently flow cytometry (3,4). Several companies currently market signal amplification systems based on this method including Perkin Elmer Life Science Products (Boston MA), Dako (Glostrup, Denmark), and FlowAmp Systems (Cleveland, OH). The last system is specifically adapted for flow cytometry.

For most applications enzymatic amplification of signal is 5-100 fold, permitting detection of low abundance antigens not detectable by standard techniques. Occasionally, >100 fold amplification can be attained. Adams compared enzymatic amplification of signal with conventional avidin-biotinylated enzyme complex (ABC)

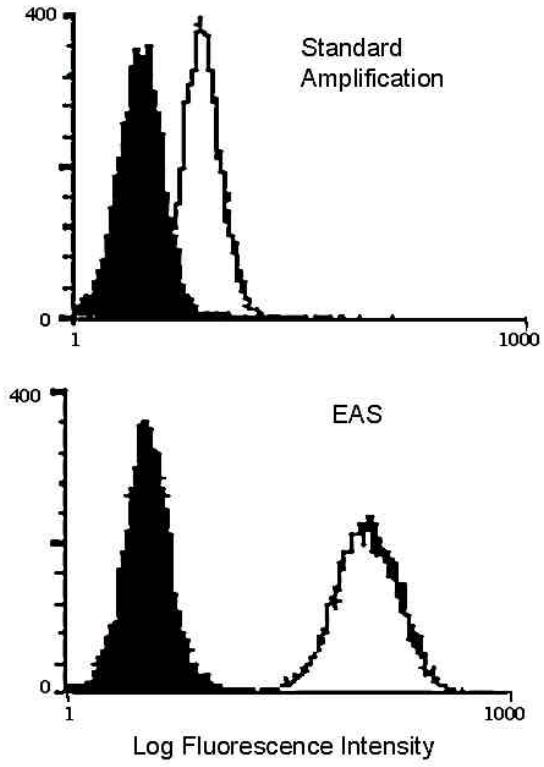

Figure 2. Enzymatic amplification. KG1a cells were stained with 1ng control IgG1 (filled histograms) or with 1ng anti-CD34 (open histograms). The cells were processed for flow cytometric analysis by a standard amplification procedure (indirect staining, upper panel) or by enzymatic amplification staining, EAS (lower panel). The results indicate that EAS enhances the signal separation between specific and control immunoglobulin by 50-60 fold compared to a standard amplification procedure.

for immunohistochemical staining of tissue and found up to a 1000-fold increased sensitivity with the amplification method (25). Merz. *et. al.* achieved up to a 10,000-fold amplification of CD20 and CD45RO detection on tissue sections (28), although this degree of amplification appears to be exceptional.

We have obtained 10-100 fold amplification of fluorescent signal with enzymatic amplification staining, as compared with indirect staining procedures (Figure 2). While we do observe a slight increase in background fluorescence associated with enzymatic amplification, this increase in noise is minimal compared with the marked increase in specific, antibody-mediated signal. The end result is that the overall signal to noise ratio is greatly increased. Moreover, the level of specific signal amplification appears to be antigen independent, having worked well in our hands for over 50 different target antigens. The procedure not only provides enhanced sensitivity but also cost savings as one can use 1/10th-1/100th the amount of antibody utilized in standard antibody staining.

Interlab variability in the extent of amplification may be due to differences in methods, cells analyzed, or dilution of reagents. The tyramide deposition reaction is very rapid, and minor differences in amplification reaction time may lead to major variations in final signal intensity (24). The concentrations of antibody and tyramide are important parameters and may require careful titration (24). An inadvertantly high concentration of tyramide or HRP-conjugated antibody may lead to preferential homodimerization of tyramide and paradoxical quenching of signal (24). In addition, antigens already present at high copy number may show little appreciable amplification due to excessive generation of reactive tyramide species leading again to paradoxical quenching (32).

One concern has been that diffusion of reactive tyramide intermediates could cause poor signal localization with artifactual staining of bystander cells (4). Earnshaw and Osbourn addressed this concern by applying enzymatic amplification staining to mixed populations lymphocytes and monocytes, using as a target antigen CD36 which is expressed on monocytes but not lymphocytes (4). Enzymatic amplification caused a 2.5 to 3-fold increment in fluorescent intensity on monocytes with no concomitant increase in fluorecence emission by lymphocytes, indicating lack of non-specific transfer of signal (4). We have used similar cell mixing studies to confirm that enzymatic amplification does not artifactually stain bystander cells (D. Kaplan personal observations).

For nucleic acid fluorescent *in situ* hybridization (FISH) enzymatic amplification staining has been applied with variable success in maintaining sharply localized signal (24,33-36). Reiterative amplification particularly seems to erode signal resolution in FISH. Many authors have used high-viscosity polymers to improve signal resolution (35-37). For example, Van Gijlswijk *et. al.* reported that polyvinyl alcohol and dextran sulfate improved FISH signal localization without diminishing intensity (35).

For immunogold-electron microscopy signal localization is of obvious importance. For this application, Mayer and Benayan evaluated signal resolution associated with enzymatic amplification staining for carbomyl phosphate synthetase, an enzyme localized along the inner mitochondrial membrane (31). They measured the distance from the mitochondrial cristae membrane to the deposited gold particles, comparing a standard method with enzymatic amplification. For both techniques the majority of label was within 10 nm of the cristae membrane, although the average distance was slightly higher for the amplified technique (11.2 nm vs 8.7 nm), indicating that amplification maintains a high level of resolution in immunogold-electron microscopy (31).

The usefulness of tyramide lies not only in its biochemical reaction characteristics but also in its flexibility in being tagged. The species used most widely for enzymatic amplification is biotinylated tyramide (24). Tyramide has also been conjugated to many fluorochromes including green fluorescent tags (fluorescein, rhodamine

green, BODIPY FL and eosin), blue tags (7-hydroxycoumarin, aminomethlycoumarin) and red tags (rhodamine red, X-rhodamine, Texas red, Cy3, TRITC, and SI-Red), allowing for multicolor analysis (24, 38). Most conjugates have performed well, with the exceptions of Texas red and X-rhodamine (38). Several of these fluorescent conjugates are available commercially from Perkin Elmer Life Science Products. Tyramide has also been conjugated with several haptens detectable with a secondary antibodies, such as digoxigenin, dinitrophenyl, and trinitrophenyl (34). Mayer and Bendayan coupled tyramide to colloidal gold tagged bovine serum albumin and conjugated the resultant molecule to immunoglobulin for use in immunoelectron microscopy (21).

Many permutations of the basic amplification process exist. Several investigators have employed serial rounds of enzymatic amplification to boost signal, using for example a biotinylated-tyramide conjugate followed by streptavidin-peroxidase followed again by a biotinylated-tyramide ampification (4, 24, 39). An extremely sensitive method called super-CARD was developed by Bhattacharya for Dot-ELISAs (40). Super-CARD enhances signal by adding electon-rich proteins (p-hydroxy proprionic acid substituted casein, gelatin or albumin) as non-specific blocking agents (40). The adsorption of these proteins next to the target antigen allows for greater deposition of tyramide conjugates during the enzymatic amplification reaction (40). The authors report an impressive degree of signal amplification with super-CARD, 10^4 to 10^5-fold as compared with conventional dot-ELISA and 5 to 10-fold as compared with standard enzymatic amplification staining (40). Super-CARD in fact permitted detection of as little as 0.1 pg/well of target antigen (40).

4.APPLICATIONS OF ENZYMATIC AMPLIFICATION STAINING

4.1. Applications other than flow cytometry

Tyramide based amplification methods have been successfully adapted to immunohistochemistry (25-28), *in situ* hybridization (24,29), immunoelectron microscopy (30,31), ELISA (22), and DNA microarray analysis (41). However, the procedure has not gained widespread use in clinical testing, possibly due either to the novelty of the method or to the effort involved in optimizing amplification. Recently, Hashizume *et. al*. described an automated platform which may facilitate generalized implementation of the amplification staining procedure in diagnostic laboratories (42).

Tyramide based signal amplification has been used successfully in immunohistochemistry in several laboratories (25-28,43-49). Examples include documenting neuroendocrine differentiation (chromogranin expression) in squamous cell lung cancers and in adenocarcinomas of the stomach (43, 44), identification of nitric oxide synthetase in myocardium (45), detection of gonadotropin subunits in pituitary adenomas (27), and documenting the co-localization of tau and ubiquitin in neurofibrillary tangles and plaques of Alzheimer's disease (46). This approach has also been used for sensitive detection of apoptotic cells (47) and of cell proliferation. (48).

In the field of hematopathology, Luo *et. al.* have used catalyzed reporter deposition to amplify the immuno-histochemical signal for CD5 (49). CD5 detection on routine paraffin embedded tissues can be difficult and is important in distinguishing small lymphocytic lymphoma and mantle cell lymphoma from other B-cell lymphoproliferative disorders (50). Luo *et. al.* were able to identify a strong CD5 specific signal in the vast majority of mantle cell and small lymphocytic lymphomas in which standard staining failed to produce significant signal (49). Malisius *et. al.* used enzymatic amplification staining to detect CD2, CD3, CD4 and CD5 in formalin fixed paraffin embeded tissues, using monoclonal antibodies to fixation-sensitive epitopes (51). Their successful staining with these antibodies suggests that formalin fixation may not quantitatively denature proteins, permitting detection of rare residual intact epitopes by highly sensitive techniques. Inagaki *et. al.* used enzymatic amplification staining to evaluate expression of low-abundance CD44 isoforms in diffuse large B cell lymphomas (52). In their analysis, CD44v6 expression correlated with inferior overall survival.

Erber *et. al.* stained bone marrow biopsy specimens with a panel of 43 antibodies, comparing enzymatic amplification with the standard method (53). They identified 8 antibodies which gave staining by the amplification but not by the standard method. These included antibodies to CD9 (P-1/33), CD16 (DJ130), CD23 (MHM6) and CD33 (WM54). Nine additional antibodies provided direct staining by the amplification technique but required antigen retrieval for the standard method. This group included antibodies to CD30 (Ber H2), CD5 (CD5/54), TdT (rabbit polyclonal), and CD79a (JCB117). In addition, enzymatic amplification permitted many antibodies to be used at much greater dilutions (200-fold) than in the standard method (53). Merz et. al., were able to stain IgM, IgD, and CD7 (IOT7) in lymphoid tissue sections using enzymatic amplification but not by standard methods (28). Therefore, enzymatic amplification may be a useful resource for immunohistochemical staining of bone marrow and lymph node specimens, particularly in detecting low abundance or fixation-sensitive epitopes.

Perhaps the most common application of enzymatic amplification staining is for *in situ* hybridization (ISH) (24). Notably, this approach provides the ability to detect single copy genes and to amplify in multiple colors (24,34,54,55). The exquisite sensitivity of this method is extremely useful in detecting viral genomes in tissue specimens. For instance, tyramide amplification has been used to detect single genome copies of human papilloma virus in cervical cells (54, 56,57), herpes virus in human breast milk (58), parvovirus in the peripheral blood (59), HTLV-1 in cerebrospinal fluid and peripheral blood cells (60), and HIV in blood cells or autopsy tissue (39). Enzymatic amplification has also been applied to ISH for detection of low abundance transcripts such as leptin (61) and cytokines (62). The enhanced sensitivity of cytokine

mRNA detection may be useful in the future for defining lymphoid cell subsets in tissues.

Several groups have adapted tyramide signal amplification to immunoelectron microscopy with excellent results (30,31,63). Mayer *et. al.* achieved optimal signal with a tyramide-immunoglobulin conjugate that was detected by colloidal gold-protein A (31). Importantly, when compared with a conventional method, enzymatic amplification did not compromise the precision of signal localization (31).

4.2. Applications in flow cytometry

4.2.1. Detection Of Antigens On Neoplastic Cells

Flow cytometry has particular utility in determining B cell clonality based on immunoglobulin light chain isotype restriction. Detection of clonal B cell populations has been hampered by low expression levels of immunoglobulin in frequent cases of B cell lymphoma. For example, surface immunoglobulin is often faint or not detected on B cells of chronic lymphocytic leukemia (CLL) (64-66). The detection of immunoglobulin light chain isotype can be enhanced by using intracellular staining (67), a procedure that is time consuming and prone to high background staining. The low expression of surface immunoglobulin impairs the ability to determine clonality of cells, often requiring the use of statistical methods such as the Kolmogorov-Smirinov test to help discriminate a positive from a negative cell population (68,69). The Kolmogorov-Smirinov test has been criticized for being too sensitive to reliably determine significant differences in cell populations (68,69). Regardless, no mathematical subtraction technique can distinguish between two cell populations without a detectable fluorescent shift.

We have used enzymatic amplification staining on clinical samples to enhance detection of surface immunoglobulin in our diagnostic procedures. By this approach, we were able to detect immunoglobulin light chain expression on many lymphoma samples in which surface immuglobulin could not be detected by standard techniques (70). This enhanced sensitivity may be prove helpful to clinical laboratories trying to detect clones in patient samples .

B-cell lymphoproliferative disorders may show weak expression of a variety of key markers including CD79b, CD22, CD23 and CD5 (66,71). CD5 is particularly critical for diagnostic purposes as its expression helps distinguish chronic lymphocytic leukemia (CLL) and mantle cell lymphoma from other small B-cell malignancies such as marginal zone lymphoma and lymphoplasmacytic lymphoma (50). Using enzymatic amplification staining, we have been able to enhance detection of CD5 and CD79b in several lymphoid malignancies from clinical samples (72). This new capacity will improve evaluation of those lymphoproliferative disorders which display borderline expression of critical markers. In one case, CD5 expression was unexpectedly detected on follicular lymphoma cells. The vast majority, but not all, of follicular lymphomas distinctively lack CD5 expression (73). Nevertheless, the enhanced sensitivity of enzymatic amplification staining could lead to novel, unexpected findings that may prompt reassessment of the accepted phenotypic profiles of lymphoid malignancies.

Davies *et. al.* used *in situ* hybridization with enzymatic amplification staining to analyze B cells from patients with multiple myeloma, addressing the controversy as to whether a small pool of clonal B-cells constitute the proliferative component of myeloma (74, 75). Employing a method known as FICTION-TSA, the authors searched for chromosomal aneuploidy, using *in situ* hybridization with tyramide signal amplification, analyzing CD20+ B-cells from several myeloma cases by multiparameter flow cytometry (74). Their analysis did not detect aneuploidy in B-cells from these patients suggesting that B-cells do not appreciably participate in the clonal disorder of multiple myeloma (74).

There are many other examples of antigens expressed at low levels in hematologic malignancies. CD2, CD3, CD4, CD7, CD8 in T cell lymphomas (76,77), CD10 in follicle center lymphomas (78), CD13 and CD33 in acute myeloid leukemia, and CD34 in acute lymphoblastic leukemia (79-82) would all show increased detection rates with enzymatic amplification staining. CD34 expression is also important for defining hematopoietic stem cells (83). Interestingly, recent studies have shown that a subset of normal stem cells express low to undetectable levels of CD34 (84). This low-level CD34 expression may cause significant problems in stem cell enumeration and may warrant analysis using an amplification staining approach.

4.2.2. Detection of antigens to define lymphoid cell subsets.

Many antigens have been examined on lymphoid cells using enzymatic amplification staining. We have evaluated B-cell expression of CD5, a marker for the unique B1 subset of B-cells (85). The low levels of this antigen on B-cells often leads to difficulty in distinguishing cells that are truly $CD5^+$ from those that are $CD5^-$ (86). Using enzymatic amplification staining, we have found that a greater number of human B-cells express surface CD5 than is generally accepted (D. Kaplan unpublished observations). This unexpected finding raises questions about the currently prevailing methods for phenotypic subdivision of B cells.

In another study, we examined T-cell expression of the interleukin-2 receptor α chain CD25, which is poorly expressed on resting T-cells and difficult to detect by standard methods (72). Several studies have shown the utility of high sensitivity flow cytometric analysis for documentation of CD25 on T cells (11). In our study, enzymatic amplification staining permitted detection of CD25 on virtually all peripheral blood $CD4^+$ T cells, whereas standard staining failed to detect CD25 on these cells (72). Thus enzymatic amplification staining allowed for more precise characterization of $CD4^+$ T cell subsets based on CD25 surface expression.

Enzymatic amplification staining has also allowed detection on cytotoxic T cells of Fas ligand, an

apoptotic factor that previously could only be identified by biologic activity (3). We used a protocol known to induce Fas ligand activity in peripheral blood mononuclear cells; conventional flow cytometry could not detect upregulation of the corresponding antigen (3). We then employed the more sensitive tyramide-based enzymatic amplification staining and observed a subset of cells (10-15%) with Fas ligand expression (3). Fas ligand activity on this antigen positive cell subset was confirmed by cell sorting (3). Therefore, enzymatic amplification staining can document the presence of molecules whose expression previously could only be inferred by bioassays.

Amplification staining may also be useful to evaluate other lymphoid cell surface antigens that are expressed at low or undetectable levels. For example, until recently CD20 was thought to be specifically expressed on mature B lymphocytes, however, low level CD20 expression has been identified on a subset of T lymphocytes (87). This finding appears to explain the existence of rare CD20+ T cell lymphomas (88). Detection of this antigen on T cells and T cell lymphomas could be facilitated by enzymatic amplification staining.

The chemokine receptor CCR3 serves as a useful marker for polarized interleukin 4 and interleukin 5-producing T-cells (89). However, the CCR3 antigen is barely detectable by standard flow cytometry on this distinct T-cell subset (89). Functionally important cytokine and hormone receptors often tend to be expressed at subthreshold levels for detection by standard flow cytometry, highlighting the need for improved sensitivity in antigen detection on lymphoid cells (90).

3.3. Detection of intracellular antigens

Detection of intracellular cytokines currently requires pretreatment of cells with an inhibitor of secretion such as brefeldin A in order for the cells to accumulate sufficient amounts of cytokine to be detectable by standard flow cytometry. However, such pharmacologic inhibitors may cause confounding global perturbations in cellular homeostasis. A more direct staining procedure that avoids pharmacologic manipulation of cells would permit examination of the unperturbed intracellular environment. Liposome-based techniques, although sensitive, are not practical for intracellular staining due to their bulk which causes poor cellular penetration.

Karkmann *et. al.* have published a protocol for intracellular cytokine detection using enzymatic amplification staining (91). They were able to detect IFN-gamma and IL-4 within peripheral blood mononuclear cells stimulated with PMA plus ionomycin (91). They showed a 10-20% increase in the cytokine positive cells detected by enzymatic amplification staining relative to the standard methods and suggested that their method could register as few as 300-400 intracellular antigens per cell (91). However, their method also employed brefeldin A treatment rather than the direct examination of intracellular antigens (91). Using a different approach, we have demonstrated the utility of tyramide-based enzymatic amplification for intracellular interleukin-2 detection both with and without inhibition of cellular secretion (D. Kaplan, personal observation).

This methodology could have practical ramifications since several routinely analyzed antigens are located intracellularly, including TDT and hemoglobin , which can be difficult to detect on a consistent basis using standard flow cytometry (92). Furthermore, Although enzymatic amplification for intracellular cytokine staining has been confined to research applications, this approach is likely to gain clinical utility in the near future. The ability of enzymatic amplification staining to overcome the weaknesses of the current intracellular staining methods may permit the detection by flow cytometry of a broad array of clinically important intracellular antigens and obviate the present need for the pharmacologic manipulation of cells.

6. PERSPECTIVE

Amplification staining technology coupled with flow cytometry allows for a significant improvement in our analytical abilities. It is very possible that this enhancement in sensitivity of antigen detection may lead to significant new findings in medicine and improved clinical diagnosis. We have shown that enzymatic amplification staining can be readily applied to flow cytometry on clinical samples.

Beyond the enhanced ability to assess clonality, the generally enhanced sensitivity associated with enzymatic amplification staining may lead to redefinition of the "classic" phenotypes of leukemias and lymphomas due to the uncovering of antigens expressed below the detection limit of standard flow cytometry. For instance, we have used enzymatic amplification staining to discover aberrant CD5 expression on an otherwise classic case of $CD10^+$ follicle center cell lymphoma. With expanded application of enzymatic amplification staining to clinical material, other novel immunophenotypes will undoubtedly come to light as well. Their significance will obviously need to be evaluated by large scale clinico-pathologic studies. The increased sensitivity is also likely to lead to clearer definition of lymphoid cell subsets. For instance, we have detected in normal control samples a much higher percentage of $CD5^+$ B lymphocytes than is generally accepted. Likewise, we have also highlighted distinct subsets of $CD4^+$ T cells based on CD25 expression. Future studies of additional antigens may lead to the definition of new and unanticipated lymphocyte subpopulations.

Finally, the ability to detect intracellular cytokines without *ex vivo* cellular manipulation may permit correlation of cytokine expression patterns with disease states. Currently intracellular cytokine staining techniques require a cell stimulation period and pharmacologic secretion blockade to enhance the sensitivity of the assay. The ability directly to assess cytokine-producing T cell subsets in peripheral blood by using highly sensitive chemokine receptor analysis, for instance, may be of particular use in both the research and clinical setting.

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