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Evaluation of Events Occurring at Mucosal Surfaces: Techniques Used To Collect and Analyze Mucosal Secretions and Cells

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The mucosal immune system is distinct from the systemic one, and it is generally considered that there exists a common mucosal system, such that immunization at any mucosal inductive site would trigger an immune response at any mucosal effector site (for a review, see references 11, 52, and 83). However, some compartmentalization does exist within the mucosal immune system (20, 34, 37, 69), and these differences (systemic versus mucosal and one mucosal site versus another) point out the importance of having appropriate tools to monitor and measure any kind of mucosal event, particularly immune responses, in addition to systemic ones.

Different reviews have addressed in details the many characteristics of the mucosal immune system and their implications in vaccine development (11, 52, 83). Although these points are key elements for anyone working in this field, the present report focuses only on techniques used to measure at the mucosal level the different changes induced by a pathogen or by immunization. In this respect, mucosal immunization can be used to reach two different goals: the induction of a mucosal response against a mucosal pathogen (or a pathogen penetrating through mucosae) and/or the induction through the mucosal route of a systemic response against a nonmucosal pathogen (for a review. see reference 68). In parallel, it is important to study the host-pathogen interactions at mucosal surfaces, regardless of any immunization, to evaluate in a proper way the efficacy of the vaccine candidates. Then, in any case, one needs to use or develop tools allowing the measurement of antigen and/or pathogen uptake at mucosal surfaces, induction of mucosal (and systemic) humoral and cellular responses induced by mucosal (or systemic) immunization, and/or induction of other changes in any component of the mucosal environment. This includes specific and nonspecific immunity, quantification of viral or bacterial loads, analysis of commensal flora, and histological changes at mucosal surfaces, etc.

Although most studies published in this field have been done with laboratory animals, and with mice in particular, important differences exist in the mucosal immune systems of mice (in particular) and human beings. Indeed, many promising adjuvants and formulations selected in animals have failed in clinical trials. As the final goal of our studies, at least in human research, is to induce and measure immunity in humans, I focus when possible on the human situation. On the other hand, if one considers the techniques used to monitor human or animal responses, these are usually quite similar, except for sampling procedures, at least in the case of small laboratory animals. I therefore address these two questions successively: (i) sampling of biological material and (ii) analysis of the specific or nonspecific events occurring at the mucosal level following infection and/or immunization.

Sampling procedures will be described, always keeping in mind that contamination with material of systemic origin (blood, serum, or circulating cells) should be minimized in order to be sure that true mucosal responses will be measured in a second step. Apart from secretory immunoglobulin A (sIgA) responses, mucosal responses are quite often more difficult to detect than systemic ones, and thus any contamination with serum or blood may introduce a significant bias in the interpretation of the results. One can take advantage of the specificity of the mucosal integrins (β7, and in particular, α4β7 or αEβ7), which allows some discrimination between systemic and mucosal cells, and phenotyping may be of great help in this respect. Moreover, one can take advantage of these characteristics and analyses can be done in some well-defined situations at the systemic level when some correlation exists with mucosal responses (60, 86). Table 1 summarizes the main issues encountered when performing mucosal analyses and the different solutions proposed in the literature that I present in this review.

Although most immunological techniques used to measure systemic immunity apply quite well to large domains of mucosal immunity, some specific tools exist to investigate immune responses occurring at mucosal surfaces. These responses can be quantified and qualified (i.e., what is the level of the response, and what kind of response is it?) and also further defined in a functional assay (i.e., what is its role?). I will separate these two aspects, although in some cases qualifying a response may also be a way to define its role (for instance, detecting IgE antibodies or specific Th1/Th2 cells). I also discriminate between humoral and cellular responses, as the tools used to measure them are usually different.

Finally, systemic and mucosal systems influence each other, and it is often very important to measure in parallel immune responses in both compartments in order to study such interactions (26, 39).

COLLECTION OF CELLS AND SECRETIONS

The first step in any analysis is to collect the starting material (fluids or mucosal cells in the present case), and this often constitutes a bottleneck, qualitatively and quantitatively. The different techniques used to sample such material are summarized in Table 2, including a selection of some relevant references. As stated above, techniques and tools in this respect are usually different for animals and humans, and I will address these two points successively.

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TABLE 1. Main issues encountered in mucosal analyses and potential solutions

Issue	Potential solution(s)
Contamination of secretions with blood or systemic cells	Apply stringent and adapted sampling procedures, quantify serum proteins or hemoglobin in secretions, phenotype cells (mucosal vs systemic integrins)
Unknown dilution of secretions	Quantify total Ig or other nonspecific protein of known concentration, quantify internal standard present in lavage buffers
Too high dilution of secretions	Concentrate samples, use appropriate devices (e.g., wicks instead of lavages), perform cellular rather than humoral analyses
Unknown site of secretion (in particular in the case of gastrointestinal secretions)	Perform cellular analyses on biopsies rather than humoral analyses on secretions
Limited amount of material or limited amount of the factors or cells of interest	Use sensitive detection methods, expand cells in vitro, perform analysis at the single-cell level
Limited availability of mucosal secretions or biopsies	Try to identify correlates in periphery

Animals (in vivo and post mortem). (i) Secretions. For laboratory animals, some techniques to collect and store secretions in vivo and allow repeated measurements of antibodies in intestinal secretions or saliva have been described (17). As degradation may occur very rapidly after sampling, it is critical to collect secretions on ice, add protease inhibitors, and store the samples at low temperature (−70°C) before analysis if analysis is delayed. Analyses can also be performed on easily collected and air-dried samples of feces and saliva, allowing easier storage not requiring refrigeration (85).

Alternatively or in addition, collection of secretions can take place after sacrifice by using absorbent wicks, which may allow a better quantitative recovery and measurement of responses (27). In fact, after sacrifice, any type of sampling can be done, but in any case contamination with blood should be minimized. This is particularly the case when doing nasal washes with mice. In that case, an iron-made rigid canula (like those used to feed mice) may be inserted and then ligated into the trachea after this organ is carefully exposed and its exterior is washed of any blood or secretions. Slowly and gently pushing buffer in the canula with a syringe allows sampling of droplets at the nostril with a pipette, and any blood contamination is then easily detected; contaminated samples are eliminated. Of course, sacrifice in that case should not be done by cervical elongation. A way to minimize systemic contamination in the organs is to collect samples after extensive perfusion of animals to maximally remove blood from the tissues (Perfext method [31]). In that case, at least 20 ml of 0.1% heparin–phosphate-buffered saline is infused into the hearts of mice after blood is drawn from the subclavian vein.

Measuring internal standards (for instance, serum albumin or total antibody responses [63–65]) in addition to specific responses is a way to estimate dilution and serum contamination of samples from animal or human origin, and this aspect is addressed further below.

(ii) Cells. It is often necessary to first disrupt and digest specimens in order to isolate cells. This digestion step is critical, in particular for organs with a low number of lymphoid

TABLE 2. Sampling techniques for analysis of mucosal responses in animals and humans

Species	Conditions	Sample[a]	Responses analyzed	Relevant references (when specifically applied to mucosal immunity)
Animals	In vivo	PBLs	Cellular immunity (antibody-secreting cells, T-cell immunity, nonspecific cellular immunity)	
		Serum	Antibodies, cytokines, other soluble factors	
		Secretions, urine, feces	Antibodies, cytokines, other soluble factors, (cells)	17, 24, 82
		Biopsies, surgery (tissues, lymphoid organs)	Cellular immunity (including establishment of T-cell clones), immunohistochemistry	
	Post mortem	All types (serum, secretions, lavages, cells, etc.)	Any type of humoral and cellular immunity	24, 27, 31, 58, 77
Humans	In vivo	PBLs	Cellular immunity (antibody-secreting cells, T-cell immunity, nonspecific cellular immunity)	34, 83
		Serum	Antibodies, cytokines, other soluble factors	47, 62–64, 68
		Secretions, urine, feces	Antibodies, cytokines, other soluble factors, (cells)	3, 7, 21, 27, 38, 41–43, 47, 57, 62–64, 69, 78, 84
		Biopsies, surgery (e.g., tonsils, gastric biopsies)	Cellular immunity (including establishment of T-cell clones), immunohistochemistry	34, 49, 70

[a] PBLs do not constitute the most appropriate source to measure mucosal immunity, but they can in some situations be composed of mucosal cells en route to mucosal effector sites. Serum IgA may be quantified as well, although its function is still matter of debate.

cells. The enzymes and conditions used have to keep cells in a physiological stage and not damage them or activate them nonspecifically. Dispase and collagenase are among the most popular enzymes used (54), but digestion is often preceded by a chopping step that can be done manually or with automated tissue choppers marketed by different companies. After digestion, a final step of purification may be required, using Ficoll gradients or equivalent reagents. This is recommended in the case of crude preparations that may induce too high a background level in the tests and thus prevent an accurate analysis, although one has to be aware that such steps often induce a high loss of cells. This can be a problem when the number of cells is initially very low, and a compromise has to be found. In the case of cells isolated from lungs, it is recommended that extensive washing of lungs be done before sampling to eliminate most of the circulating cells, which are very abundant in this organ. This can be done by carefully infusing the right part of the heart with sterile phosphate-buffered saline (the lungs turn white). Of course, in any case of sampling material for examining cellular immunity, working under a hood in sterile conditions is recommended if possible.

Humans. (i) Secretions. For humans, different tools exist to collect a large number of secretions (for instance, tears and saliva) (47), and different companies market specific devices to collect and store individual samples. These devices are used routinely in clinical trials. Rectal and vaginal secretions can be collected as well, using marketed rectal absorbent wicks, for instance (35, 43). As for animal secretions, it is recommended that secretions be stored at −70°C if analysis is delayed.

The choice of the sampling method may influence the results obtained in the end. For instance, different types of saliva secretions (mixed saliva, parotid saliva, submandibular saliva, crevicular fluid, and minor [labial] gland secretions) can be sampled and analyzed separately (63, 64, 84). Devices such as specific cups, for instance, can be used to collect parotid saliva (78), while mixed saliva can be collected with absorbent wicks (Salivette or Omnisal devices). It has been observed that the salivary IgA concentration could be influenced by the saliva collection method. When comparing different ways to obtain saliva (spitting, suction, or Salivette), it has been observed that IgA concentrations were significantly lower in saliva collected with the Salivette device than in saliva collected by the suction or spitting method (3). Saliva can be collected with or without prior stimulation. In the latter case, the salivary flow rate can be increased by repeated application of a 3% citric acid solution to the rims of the tongue (57) or measured after a physiological stimulus such as lunch or effort (57, 78). Generally, if one looks for an overall IgG or IgA response in saliva, mixed saliva is an appropriate secretion, while it worth sampling parotid saliva if, for instance, IgA subclasses are to be measured.

Nasal collection methods were also shown to influence the measurement of eosinophil cationic protein, with the method based upon absorption or nasal washes being the best (42).

Comparisons have also been made between lavages and wicks to recover cervicovaginal secretions in humans (62), and it was found that Ig and protein concentrations in lavage samples were generally over 100 times lower than those in secretions captured directly from mucosal surfaces with wicks or similar tools. It thus appears that in cases where too high dilutions may prevent detection of immune responses, wicks would be preferred. However, the use of such devices should in no case damage mucosa and allow recovery of systemic fluids or blood. The use of ophthalmic sponges, for instance (7), minimizes such risk and allows recovery of fluids (cervicovaginal fluid or saliva) in which cytokines could be successfully identified. As stated before, systemic contamination and dilution of the sample has to be addressed in a second step (see Analysis of Mucosal Responses below), and in cases where washes are performed, a possibility is to use directly a washing liquid that contains lithium as an internal reference (41). Dilution factors and subsequently concentrations in undiluted secretions can thereby be calculated.

These studies highlight the importance of choosing the right method to get the right answer to the right question before initiating any study. Preliminary bibliographic research should be done, as no absolute method of sampling exists and the method instead should be chosen according to the parameter to be evaluated.

Finally (and perhaps most important), the pertinence of sampling some secretions or extracts, particularly fecal extracts (24, 73), has been questioned by some authors (22), where responses in fecal extracts were compared to those obtained with whole gut lavage fluid of the same patients. Those authors observed that immunological tests on saline extracts of feces did not represent the true status of the gut humoral immune system and strongly discouraged such studies. It is in part due to such discrepancies that techniques allowing direct sampling and analysis of cells have been developed.

(ii) Cells. Cells in humans can be obtained from biopsies (for example, gastrointestinal [12, 40]) or after surgery. Normal tissues surrounding tumors may be a convenient source to obtain mucosal mast cells and perform functional allergy tests in the case of lung tumors, for instance. Tonsils, which are commonly sampled in children, are also a convenient source to study cells such as B cells and antigen-presenting cells (APCs).

Some secretions (bronchoalveolar or intestinal lavages, for instance) may be a source of immune cells, and once obtained, they can also be analyzed by conventional techniques used for measuring cellular immunity. It may actually be of interest to sample cells present in secretions with harmless devices (avoiding biopsies or surgery), such as cytobrushes. Reports of studies carried out with human immunodeficiency virus (HIV) have described the use of such devices (38). The device can be inserted into the cervical os, gently rotated, and then transferred into medium (a few milliliters). To avoid contamination with blood, the cytobrush has to be used before other sampling and has to be rejected if it contains visible blood. After agitation, cervical mononuclear cells can be isolated by use of Ficoll-Hypaque gradients. Reported yields were between 8×10^5 and 1.2×10^6 cervical mononuclear cells for one sample and allowed subsequent analyses (phenotyping and enzyme-linked immunospot assays [ELISPOTs]).

Finally, sampling of cells can be done to perform subsequent reverse transcription-PCR (RT-PCR) analysis (with animals too, of course). In that case, the way samples are collected is critical in order to avoid RNA degradation. An alternative to immediate freezing in liquid nitrogen is to use commercial buffers allowing prevention of RNase activity and storage at room temperature or 4°C before extraction of RNA.

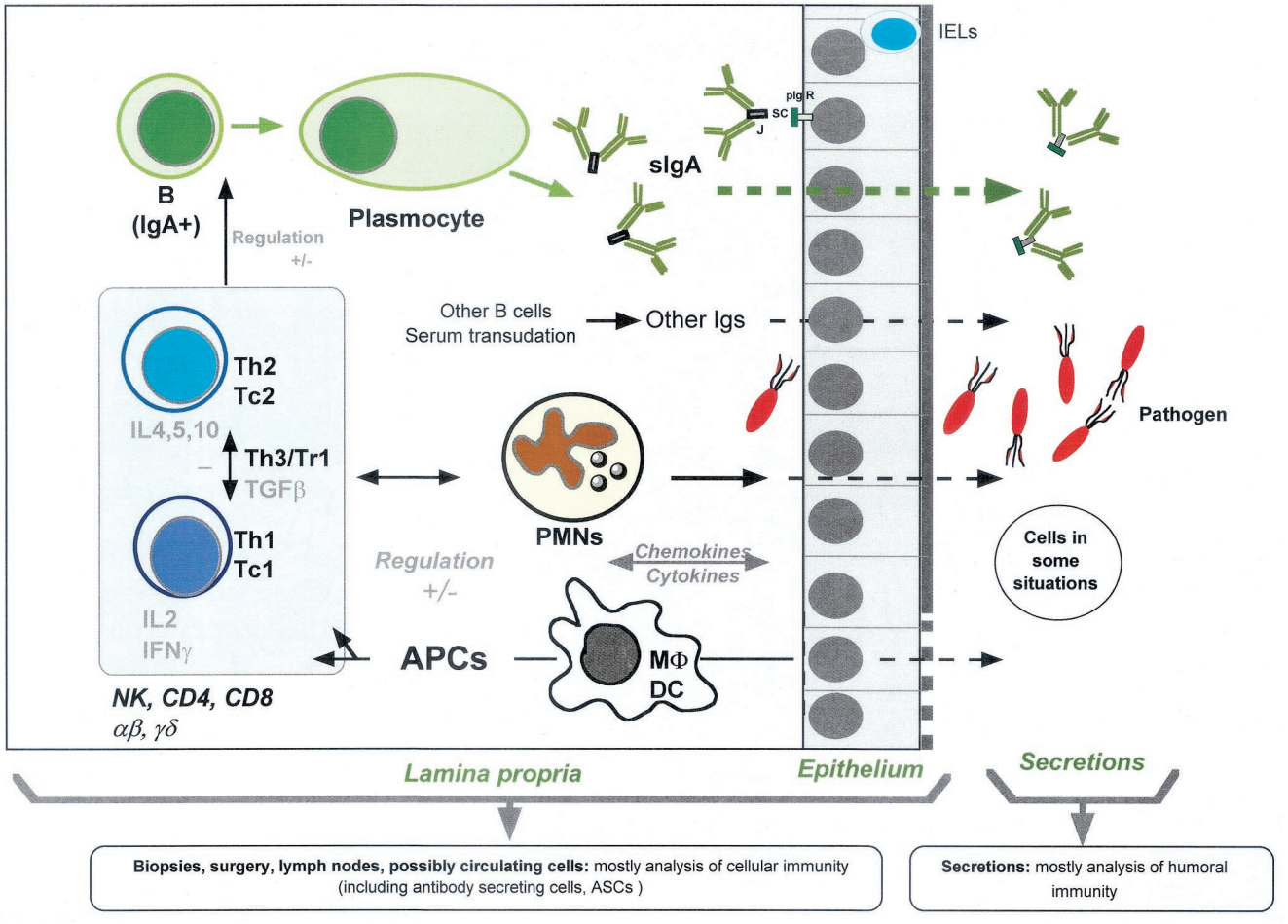

FIG. 1. Cells and soluble factors present within mucosal tissues and secretions. Cellular analyses are performed mostly on material coming from biopsies or surgery, while soluble mediators (antibodies, cytokines, and chemokines) are preferentially measured in secretions. Some cells may also be present in the lumen, and their proportion may be higher under certain physiological conditions (the hormonal cycle in genital tissues) or pathological conditions (inflammation or rupture of epithelium integrity by pathogens). NK, natural killer cells; IELs, intraepithelial lymphocytes; Mϕ, macrophages; SC, secretory component; PMNs, polymorphonuclear leukocytes; IL, interleukin; TGFβ, transforming growth factor β; IFNγ, gamma interferon.

ANALYSIS OF MUCOSAL RESPONSES

Figure 1 presents different components of the immune response potentially present in mucosal effector sites (some of them are potentially present in inductive sites as well, although in different types or stages and proportions), both in the mucosa and in secretions, and Table 3 presents some of the different techniques available to evaluate them. As techniques used to monitor responses in humans and animals do not usually differ except for specific reagents, they are described together in the following paragraphs.

Humoral responses. (i) Qualification and quantification. Once collected, centrifuged, possibly filtrated, and diluted in appropriate buffers, samples are then analyzed by conventional techniques. For instance, all isotypes can be measured in secretions, but the most commonly analyzed is the IgA isotype. In addition, detecting the presence of the secretory component may help to confirm the nature of the IgA detected (monomeric or dimeric), as only the secreted dimeric form (sIgA) bears this peptide. Alternatively or in addition, high-pressure liquid chromatography techniques may allow discrimination between monomeric and polymeric IgA.

The need to quantify dilution and contamination can be addressed by different means. First of all, specific activity can be normalized to the total level of the corresponding isotype present in the same sample (65). This allows calculation of specific activities, according to the formula specific activity = specific Ig/total IgG or IgA. It is also important to compare, in the same assay, the values obtained to those of reference standards, usually purified gamma globulins or pooled sera (65). In addition, it was also demonstrated that mucopolysaccharides in mucosal samples did not interfere with antigen-antibody recognition in enzyme-linked immunosorbent assays (ELISAs), by adding and quantifying in a second step known amounts of specific IgG or IgA (65). Another way to measure dilution in samples induced by lavage is to add to the washing buffer a known product such as lithium as an internal reference, as mentioned before (41).

Contamination by serum transudation is also a very impor-

TABLE 3. Tests and techniques used in mucosal immunity (humoral and cellular)[a]

Parameter(s) studied	Qualification and quantification		Functional assays	
	Test(s)	Reference(s) (when specifically applied to mucosal immunity)	Test(s)	Reference(s) (when specifically applied to mucosal immunity)
Humoral immunity (soluble factors, specific and nonspecific)	ELISA (antibodies, cytokines)	22, 31, 63–65, 72, 86	Hemagglutination inhibition, seroneutralization	37, 53
	Western blotting		Antibody-mediated passive protection	65
	Immunoprecipitation		Inhibition of binding	
	Immunodiffusion		Inhibition of transcytosis	40
			In vitro toxicity	75
			Sensitization or allergic reaction	72
			Opsonophagocytosis	
Cellular immunity (specific and nonspecific)	ELISPOTs (antibodies, cytokines)	2, 9, 10, 18, 20, 26, 32, 36, 44, 51, 54, 60, 71, 72, 77, 81, 82	CTLs	59, 70
	Intracellular cytokine staining	4	DTH (Th1)	79
	RT-PCR	6, 50, 57, 88	APC function (DCs)	30, 74, 76
	Isolation of T-cell clones	12–14	Allergic reaction (role of mast cells)	29, 61
	Detection of APCs	15, 76		
	Lymphoproliferation	28, 38	Chemotaxis assay	30
	Immunohistochemistry	16, 72	Development of mucosal system (role of cytokines, use of transgenic mice)	45
	Phenotyping fluorescence-activated cell sorter analysis (receptors, αβ/γδ integrins, costimulatory molecules, etc.)	16, 70	Cell-mediated passive protection	56
Others study of mucosal pathogens [including protection assays], study of microflora, biodistribution, mucosal penetration, etc.	Pathogen or microflora identification and quantification	25, 41, 46, 48, 53, 58, 66, 74	Enzymatic assays	23, 25, 48
	Histology (mucosal modifications)	1, 19, 48, 72, 80, 87		
	Use of in vitro mucosal chambers (study antigen or pathogen uptake and induced phenomena)	55	Use of in vitro mucosal chambers (study antigen or pathogen uptake and induced phenomena)	55

[a] The separation between (i) qualification and quantification and (ii) functional assays may seem artificial in some situations, but this has nevertheless been attempted for more clarity.

tant point to consider. In the study using the Perfext method (31), the authors addressed this question by comparing the mucosal tissue/serum antibody titer ratios after immunization by different routes with those obtained after passive intravenous immunization using immune serum with a known titer. They observed that local responses exceeded those in serum and could thus not be explained by transudation of serum antibodies. In addition, they observed that only a few percent of the passively administered antibodies were found in the mucosal tissues. However, this may not always be the case, in particular when measuring mucosal responses that are not fully efficient, and it is important, in particular with humans, to quantify the extent of serum transudation in mucosal secretions if possible. This can be done by measuring the amount of human serum albumin (HSA), since this protein originates exclusively in serum, and it was chosen with the assumption that diffusion of serum IgG and IgA through the mucosae was similar to that of HSA (64). Titration of this protein allowed those authors to calculate a relative coefficient of excretion (RCE) according to the formula (Ig in fluid/HSA in fluid)/(Ig in serum/HSA in serum). The RCE can be calculated either for total Ig or for specific Ig. An RCE significantly greater than 1 indicates local production, and RCE values equal to or near 1 suggest transudation from serum. Contamination with blood can also be addressed by measuring hemoglobin with commercial kits (Sigma). This allows the identification (and elimination) of samples presenting too high values of hemoglobin due to mucosal damage (caused, for instance, by the sampling procedure) or for any other reason.

Apart from antibodies, any soluble factor can be measured

in a secretion, as long as a sensitive enough assay exists. For instance, cytokines have been successfully detected in saliva or cervical washes (7). However, one has to be aware of potential drawbacks when measuring cytokines, due to their high sensitivity to proteolysis or denaturation, in particular in secretions.

(ii) Functional assays. Any functional assay using serum can in theory be performed with mucosal secretions, depending of course on the concentration of the active factor. Below are some examples of tests that can be performed with mucosal antibodies.

(a) Allergy (antibody arm). Allergic reactions need antibodies and allergens on the one hand and cells on the other. Antibodies (mainly IgE) present at the mucosal level may be used in such in vitro assays, given that effector cells are available and can be sensitized by such antibodies before challenge with allergen. Alternatively, challenge with allergen can also be done in vivo, and subsequent measurement of IgE or cytokine (interleukin-4 and -13) transcripts can be done by RT-PCR, showing their involvement in allergic reactions (6) (see "Measurement of cellular responses" below).

(b) In vitro viral neutralization. IgA antibodies have been shown to neutralize flu virus inside epithelial cells (53). In that work, antihemagglutinin (anti-HA) polymeric IgA (representative of dimeric mucosal sIgA), but not anti-HA IgG, delivered to the basolateral surface colocalized with HA within the cell and induced a reduction in viral titers (in supernatants and cell lysates). IgAs were demonstrated to neutralize virus inside the cells and not in the extracellular medium.

Intracellular neutralization and inhibition of transcytosis has also been demonstrated more recently in the case of HIV, still using dimeric (anti-HIV) IgA (5). Neutralization occurred intracellularly within the apical recycling endosome, and immune complexes specifically recycled to the mucosal surface.

(c) Passive protection. Such polymeric IgAs have also been demonstrated in the case of flu to mediate passive protection against viral challenge upon intravenous administration, while IgGs were unable to do so (66). Specificity of the protection was also confirmed by administration of anti-IgA antibodies, which eliminated protection.

(d) Inhibition of in vitro toxicity. IgA antibodies may also be assessed for their ability to prevent tissue damage mediated by bacteria or their toxins. This has been done, for instance, in the case of *Clostridium difficile* (75), where polymeric IgA was demonstrated to be more efficient than monomeric IgA or IgG of the same specificity in preventing damage induced in cell culture by *C. difficile* toxin A. Such tests can in theory be carried out with any toxin from a given pathogen if one has a relevant cell culture.

The examples presented above (viral neutralization, passive protection, and inhibition of toxicity) refer to studies in which IgA antibodies (monoclonal, monomeric or polymeric) were used versus IgG and considered effectors of mucosal immunity. However, natural secretions were not used as such. When using high doses of antibodies as in these tests, one has to carefully quantify the reagents used, and any extrapolation to the natural situation and physiological concentration at the mucosal level has to be justified and discussed.

Measurement of cellular responses. Although the most characteristic and popular mediators of mucosal immunity are secretory IgAs, they are definitely not the only component of the local immune response. Even when sIgA identification and quantification are of importance, one has to consider that production of these antibodies represents only the final step of a complex process. In addition, the quantification of antibodies or other soluble mediators accumulated in secretions such as cytokines presents some limitations: it brings little information on the dynamic aspects of immune responses and, in particular, is unable to yield information concerning the precise anatomical location(s) of antibody or cytokine formation. The analysis of secretions at the cellular level is more informative in this respect. All in all, it is of great interest to study the mechanisms of antigen presentation (for instance, T- and B-cell regulation or activation of nonspecific cells), among other important parameters at mucosal surfaces. Although they are less easy to perform than tests using secretions because of the sampling step, tests measuring cellular immunity are being used more and more by those working in this field. In fact, once the cells are obtained in sufficient number, any test measuring cellular immunity can be performed, and some examples of such techniques applied to mucosal responses are presented below.

(i) Qualification and quantification. (a) ELISPOT (antibodies and cytokines). Since its original description (9), the ELISPOT technique has been employed as an alternative to conventional plaque-forming cell assay to enumerate specific as well as total Ig-secreting cells and also to detect a variety of cells (lymphoid or non lymphoid) secreting factors with immunological properties, such as cytokines (54). Antigen (against which antibodies are directed) or capture antibodies (directed against a cytokine, for instance) are adsorbed onto a solid surface, and in a second step the cells to be analyzed for secretion are added to the wells. After a variable time of incubation depending on the experiment, cells are removed by the use of a detergent (Tween, for instance), and subsequent steps are similar to those used in ELISAs, except for the final revelation step. The secreting cells are then visualized as spots that are then counted under a dissecting microscope. The appearance of spots is different from that of the background, and counting them usually does not constitute a difficult step for someone with some experience. However, it is often time-consuming, and some companies now propose the use of devices for automated counting.

The method can be employed to detect secreting cells in suspensions prepared from a variety of animal species (for which reagents exist) and from different anatomic compartments. As stated above, the limiting factors are again the source of cells and the recovery yields. When working on animals, collection of different organs may be rather easy after sacrifice, and this usually allows recovery of significant numbers of cells after appropriate digestion. For humans, the source of cells is usually biopsies or samples obtained after surgery, and in the former case the yields are usually low whatever the technique used to obtain the cells, which may constitute some limitation.

A considerable number of studies have used this technique to measure in particular IgA secretory cells in nasal tissue, salivary glands, Peyer's patches, lamina propria, lymph nodes, or spleen (2, 18, 32, 54). Similarly, cytokine-secreting cells have been detected in different mucosal inductive and effector sites, in both animals and humans (36, 54, 77). Spontaneous cytokine secretion can be monitored or, alternatively, such secretion

may be quantified after specific antigenic stimulation. The ELISPOT technique can be used with different revelation systems in order to visualize different cells secreting, for instance, different isotypes in the same well (10). It can also be combined with specific purification steps (immunobeads) before incubation (44).

Apart from mice, this technique has been used with a large variety of animals from mice to monkeys (81, 82) and, of course, with humans, for detection of IgE to gamma interferon (in gastric biopsies, for instance) (20, 28, 36, 51, 71). However, due to the sometimes limited availability of biopsies, some authors have correlated the detection of antibody-secreting cells in the blood circulation with the level of mucosal antibody responses (60). Actually, when stimulated in inductive sites, cells bearing a mucosal addressin will, over a short window of time (usually about 1 week after induction in mucosal sites), transit through the blood circulation, where they can be picked up and quantified (34, 86). This may constitute a convenient alternative to the use of biopsies when schedules of immunization and sampling are well defined and when a correlation between local and peripheral detection within this interval of time has been clearly demonstrated.

(b) Intracellular cytokine staining. As it is of interest to phenotype the very cells that produce cytokines, the use of intracellular cytokine staining may also be applied to mucosal cells. Indeed, this has been done with gastric biopsies from infected volunteers (4).

(c) RT-PCR (cytokines, chemokines, and other antigens). A very sensitive way to measure protein expression (cytokine expression in particular) is to use RT-PCR. Given the usually small amount of material obtained from mucosal biopsies or secretions, this technique is of particular interest in this context. As stated in the sampling section above, the way samples are collected is critical in order to avoid RNA degradation. RT-PCR cytokine detection has been described in reports of different studies using biopsies as a starting material, including studies performed with animals (for example, using nasal tissue [6, 50]) and humans (for example, *Helicobacter pylori* in gastric biopsies [88]). In addition, RT-PCR can be used to detect direct expression of antigens from pathogens, such as *H. pylori* in dental plaques (58). A similar approach is currently being taken in our department with gastric biopsies, addressing in parallel the expression of cytokines and of different *H. pylori* antigens (B. Rokbi et al., unpublished data).

(d) Expansion of T-cell clones from biopsies. Although the amount of lymphocytes in human biopsies is usually small, some T-cell expansion can be carried out and can allow subsequent analysis using conventional T-cell immunology techniques. Actually, techniques used to generate clones from peripheral blood lymphocytes (PBLs) (13) have been applied to biopsies from bronchial and nasal mucosae or from gastric mucosa (12, 14). Once obtained, clones can be characterized, and their antigenic specificity, phenotype, and cytokine profile can be determined by using autologous Epstein-Barr virus-transformed cells as APCs. One drawback of these techniques is the use of clones resulting usually from a nonspecific polyclonal activation, which in most cases constitutes the first step of the cloning procedure. This may preferentially select some more resistant or activatable clones, regardless of their Th profile or antigenic specificity. We are carrying out similar experiments in our laboratory and have observed that the use of human serum and of culture medium specifically designed for human cells dramatically increased the number of clones that we could get from each biopsy (unpublished observations).

(e) APCs and nonspecific immunity. Apart from lymphocytes, identification and quantification of APCs is of interest. Among them, dendritic cells (DCs) are of critical importance, and a growing number of papers highlight the facts that mucosal DCs are different from peripheral ones and that their interactions with mucosal T cells have different consequences. In fact, induction of tolerance is one of the hallmark of mucosal inductive sites, and APCs play a major role in this respect, as indicated by, for example, studies carried out on conjunctival mucosa (15) or respiratory tract DCs (67, 76).

In addition, characterization of cells such as phagocytes, in particular resident neutrophils or mast cells, is of critical interest in some pathologies and has to be addressed, possibly by histology or fluorescence-activated cell sorting techniques.

(f) Immunohistochemistry, flow cytometry analysis, and more. Techniques used to analyze immune responses in tissue sections apply quite well to the analysis of mucosal immunity. Although the ELISPOT technique is often preferred for the detection of antibodies or cytokines, immunohistochemistry can be used to analyze different cell markers, in situ for instance (16, 72, 80). This can be combined with flow cytometry analysis.

(ii) Functional assays. As for secretions, in theory any functional assay in cellular immunity may be applied to mucosal cells, as long as these cells are in sufficient number and in appropriate physiological conditions and status. Below are some examples of cellular functional tests carried out with mucosal or any other type of cells obtained from biopsies.

(a) CTLs. Pulmonary cells or cells isolated from mediastinal lymph nodes have often been analyzed in cytotoxic-T-lymphocyte (CTL) assays in the flu model (59). As mentioned before, when cells are isolated from lungs, their local status has to be checked. Other sites may constitute sources for cells presenting CTL activity. $CD8^+$ cells have been expanded from intestinal biopsies of HIV-infected volunteers and characterized by their expression of the mucosal integrin CD103/$\alpha E\beta 7$ (70). CTL activity has been measured after expansion of these cells, showing that this kind of test can be carried out, even from a limited starting material.

(b) DTH. Apart from CTL responses (usually CD8 mediated), one can address the functionality of resident CD4 Th1 cells in tests such as delayed-type hypersensitivity (DTH). This has been done using nasal CD4 cells in mice (79), in which such cells were demonstrated to mediate DTH, and their presence was associated with protection against flu. Nasal tissue is highly vascularized, and once again when carrying out such studies, the mucosal nature of the cells has to be checked and sampling methods have to be carefully chosen.

(c) Role of APCs. DCs or other APCs isolated and characterized as mentioned in the preceding section can also evaluated in functional assays (antigen presentation to T cells, for instance; for a review, see reference 76). The role of DCs in pathogen propagation as in the case of HIV has also been addressed at the mucosal level (74).

(d) Nonspecific immunity and mast cells. The role of nonspecific cells is often critical at the mucosal level (phagocytes,

including neutrophils, or cells such as mast cells in allergic reactions). In this respect, mucosal mast cells (from intestinal biopsies or lung surgery, among other sources) may be sensitized with IgE of known specificity and concentration before challenge with allergen or stimulated directly with other compounds. Release of mediators can then be quantified (29). This constitutes an evaluation of the cellular arm of an allergic reaction, whose humoral arm was already considered in the preceding section. In addition, the direct role of mast cells in protection can also be addressed at the mucosal level (61).

(e) Chemotaxis and chemokines. It may be of interest to investigate the presence of chemotactic substances at the mucosal level. These chemokines may be induced by infection and produced by epithelial cells or other subtypes. These substances can be quantified by classical techniques like ELISA and also can be tested in in vitro chemotaxis assays. In such assays, one can monitor the induced migration of different cell types, and mucosal cells have been evaluated in such assays (30), using, for instance, DCs isolated from Peyer's patches. These studies allowed the phenotyping of different subsets of DCs and correlation of their differential migration to their chemokine receptor expression (CCR6 versus CCR7).

(f) M-cell differentiation and function. Assessment of the direct functionality and development of natural intraepithelial or M cells is a difficult task (45), and a study allowing in vitro differentiation of Peyer's patch lymphocytes by cocultivation with the Caco-2 cell line has been helpful in this regard (40). Such a method allowed the lymphocyte-induced reorganization of the epithelial monolayer and the assessment of bacterial (*Vibrio cholerae*) or antigen uptake and transport through neodifferentiated M cells. Such a model would in theory allow the study of antigen uptake and guide investigators in the search for optimal mucosal formulations (drugs or vaccines), as long as a direct link with these in vitro tests can be done with the in vivo situation (33).

(g) Passive transfer of protection against mucosal diseases. Passive transfer of protection can be done with antibodies and also with cells. This is usually done with cells of systemic origin due to the usually high number of cells required in such assays. Alternatively, mucosal T-cell expansion may give sufficient material. In any case, systemic cells can be evaluated for protection against mucosal pathogens, as has been done in the *H. pylori* model, for instance (56).

Other responses and events. (i) Qualification and quantification. (a) Quantification of pathogen load and study of microflora. In challenge studies, usually the most sensitive way to assess protection is to quantify the number of viruses, bacteria, fungi, or parasites present in or on mucosal tissues. This requires tissue or secretion sampling, followed by quantification. This has been done in such pathologies as flu (nasal tissue [53, 66]), HIV (different mucosal tissues [74]), *H. pylori* infection (gastric or oral tissues [21, 25, 58]), or urinary tract infections (46). It should be noted that a large proportion of pathogens present at mucosal surfaces are often entrapped in the mucus or below it and that when only secretions or superficial layers of mucus are sampled, this presents a clear risk of false-negative results (overoptimistic in the case of vaccination). It is generally more sensitive to sample whole tissue biopsies and to homogenize them (without damaging the pathogen) before quantification (25).

The microflora can also be qualified and quantified as well, in order to study any change induced by treatment, procedures (such as nasal intubation [87]), or immunization or to link the presence of one or another type of commensal bacteria to different biological activities. IgA protease-producing bacteria in human nasal mucosa were analyzed (41) for the respective roles of *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Streptococcus mitis*.

(b) Histology and analysis of mucosal modifications. Histology can be performed to study any modification or damage induced by immune responses, treatment, and/or mucosal pathogens (1, 48, 80). These techniques are then usually combined with other tests in the same study (e.g., detection of immune responses or bacterial load), in order to establish correlations if possible. Lung damage induced by allergic reactions can be measured in the light of IgE responses, for instance (72). Damage induced by nasal intubation may also be assessed by histology (87), as can changes induced by immunotherapy (8, 19). This type of study should also be done when testing new mucosal adjuvants and formulations, in which adjuvanticity may also be linked to local toxicity (for a review, see reference 68).

(c) In vitro mucosa. It may also be of great interest to use in vitro-reconstituted mucosa to have an easy way to monitor any change occurring at mucosal surfaces and to be able to perform kinetic studies on released mediators. For example, work has been done on nasal mucosa (cell lines or excised nasal epithelium; for a review, see reference 55). Nasal chambers of different types allowing study of the influence of other parameters such as humidity and airflow have been designed and optimized (J. Turner and Oya Alpar [Aston University], personal communication). These devices can be linked to confocal microscopy, allowing kinetic studies of antigen uptake.

(ii) Functional assays. The presence of bacteria or cells may also be measured by their enzymatic activities (41, 42, 48), but one has to be aware that because the sensitivity of the enzymatic assays is sometimes not high enough, this kind of test may need to be reserved for qualification rather than quantification of pathogens (23, 25).

CONCLUDING REMARKS

Mucosal immunity is a rapidly moving and growing field and I do not pretend to have described or even listed all techniques used in this discipline. Each technique has to be selected and developed according to the specific model and antigen(s) used. The present review has only described some examples of the most widely used techniques, focusing on the more practical and sensitive ones, and constitutes a source of references rather than a detailed description of each test and protocol.

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