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THE EFFECT OF THE INTESTINAL MICROFLORA ON SYSTEMIC DELAYED HYPERSENSITIVITY

Authorised transcript of a lecture by

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I am pleased to speak to you today about delayed hypersensitivity (DH). Having spent ten years at the Trudeau Institute, prior to joining North Carolina State University, delayed hypersensitivity has been closely tied with cellular immunity to bacterial infections throughout my work and that of my colleagues (*Carter*, 1975; *Carter* and *Collins*, 1974a, 1974b; *Carter* et al., 1980; *Carter* and *Pollard*, 1971; *Collins* and *Carter*, 1980; *Lagrange* et al., 1975; *Lagrange* and *Mackaness*, 1975; *Lane* and *Unanue*, 1972; *Lev* and *Battisto*, 1970; *MacDonald* and *Carter*, 1978, 1979, 1980, 1981; *Mackaness*, 1969). Delayed hypersensitivity is classically determined, through the work of many people during the heyday of tuberculosis (TB), primarily, the 1930s and 1940s, as the sensitivity exhibited by TB infected animals and people to purified proteins derived from the TB bacillus (PPD). TB, as a plague in the western world, caused many investigators to study the immune response to the tuberculosis organism in great detail. Through such studies it was determined that there is a sensitivity to bacterial antigens, specifically, in this example, to TB proteins, that is not immediate and evanescent as is observed in some bacterial infections but appears later in time after challenge, and remains for a longer period. The main observation in delayed hypersensitivity (DH) is swelling or induration of tissue soon after injection of the antigen, followed by subsequent decrease in that swelling, and an increase again by 24 hours. In fact, at 48 hours the injected tissue will still be indurated, whereas, with other antigens which induce a strong antibody response, the short swelling or induration following injection of the foreign antigens will disappear and remain low. Most of the swelling is due to interstitial fluids but in DH there is a characteristic influx of mononuclear cells, T-cells and macrophages, but also some neutrophils. In other types of hypersensitivity responses, basophils can be important infiltrators and you have an induration and inflammation that is mediated primarily by inflammatory products from granulocytes with a less cellular infiltrate.

Purists would say that delayed hypersensitivity is only defined in the guinea pig and particularly the guinea pig infected with mycobacteria and then elicited with PPD. Mackaness and others at the Trudeau Institute (*Collins* and *Carter*, 1980; *Mackaness*, 1962, 1964, 1969; *North*, 1973), and at other institutions throughout the world, have shown that in fact there can be a delayed hypersensitivity response to a number of purified bacterial proteins in a number of infections, primarily those caused

by facultative intracellular pathogens. A number of workers have, therefore, gone beyond using the guinea pig TB model to using other animal species. Both the footpad and the ear are very good testing sites in the mouse. The skin of the mouse and even that of the rat is not as good, for these sorts of studies, as skin of the guinea pig, or even the human, and the footpad or the ear are the preferred sites. We have used the mouse model to study the immune response to *Listeria monocytogenes*, as well as the immune response to a number of other antigens, both infectious organisms and non-infectious agents, and inanimate agents such as sheep red blood cells (SRBC). The work which I will present today was done at the Trudeau Institute and at North Carolina State University in association with Tom MacDonald, Steve Simkins, Frank Collins and Roger Brideau.

The relationship between delayed hypersensitivity and antibody production has long been suggested to be inversely associated. That is, if you have a situation in which there is a significant serologic response, then the delayed hypersensitivity response is observed to be diminished. That is, when antibody titre rises, then the DH which is elicited is diminished. It has been observed in a number of cases, in fact, that as soon as antibody is produced, we see a diminishment of DH, but in certain circumstances, when there is a very poor antibody response, those are the situations in which DH is most easily demonstrated. In fact, this posed a problem for Philippe Lagrange, now in Paris, and George Mackaness when they were together working on the definition of DH to such inanimate antigens as SRBC (*Lagrange* et al., 1975; *Lagrange* and *Mackaness*, 1975). They showed, in support of the earlier theory of Sam Salvin at the University of Pittsburgh, that DH probably is the initial response to any antigenic stimulus and only in certain circumstances is the response extended to include antibody. You can have a very short DH response because antibody appears early and therefore DH is less easily detected. In fact, if you block the antibody response with cytoxan (*Lagrange*, *Mackaness* and *Miller*, 1974), you get an even greater demonstration of DH to mycobacterial or other microbial antigens or even the sheep red cell, which again supports the view that delayed hypersensitivity and antibody responses are inversely related.

With Tom MacDonald, we did a study of the influence of the intestinal microflora on the development of delayed hypersensitivity. There had been some concern through the 1960s that, in fact, the gnotobiotic or germfree animal was unable to exhibit delayed hypersensitivity. This was primarily due to the work of Mier Lev and Jack Battisto in New York, in which they infected germfree guinea pigs with mycobacteria and then tried to elicit, in a standard, accepted test with PPD, a delayed hypersensitivity response and were unable to do so (*Lev* and *Battisto*, 1970). Other workers in Japan, Ueda, his co-workers (*Ueda* et al., 1973; 1975), and others (*Julianelle*, 1942) using mice showed that, with renewed immunisation, it was possible to demonstrate only very low levels of DH to PPD, following exposure to mycobacterial antigens, or reactivity in other tests for cell-mediated immune responsiveness (*Seicastava* et al., 1976; *Wostrnann* et al., 1970). Since it had been well demonstrated by many groups that the antibody response of gnotobiotic animals to antigenic stimulation far exceeds that of conventional animals (*Fidler*, 1975; *Hooijkaas* et al., 1984; *Kiuchi* et al., 1972; *Nielsen* and *Friis*, 1980; *Pollard* and *Nordin*, 1971; *Wostmann* et al.,

1970; *Yasutake*, 1977), we considered that the exceptionally profound serologic response of the gnotobiote might be causing the blockage of DH responses, which Merrill Chase showed decades ago to be mediated by cells rather than serum components (especially antibody).

When Tom MacDonald came to the Trudeau Institute in 1976, we were concerned about this apparent inability of germfree animals to mount a normal T-cell response. One of the first studies we performed was with skin sensitising agents (*MacDonald* and *Carter*, 1978). Although the skin sensitising agents picryl chloride and oxasolone can be argued as not being good models for DH responses of the type that mycobacteria elicit, since the response is basophil mediated, it is mediated by cells. Nonetheless, we were able to show, using either incorporation of tritiated thymidine as an indication of DNA synthesis in the draining lymph nodes or the swelling of the ear following elicitation of sensitised germfree mice, that we could sensitise a germfree animal and, just as in sensitised conventional mice, obtain very measurable and profound delayed responses. This study was followed by the SRBC work which we published in the Journal of Immunology (*MacDonald* and *Carter*, 1979), building upon the work of Lagrange and Mackaness (*Lagrange* et al., 1975; *Lagrange* and *Mackaness*, 1975). Basically, it is observed that the germfree (GF) animal does indeed exhibit a lower response, a much lower response, in the development of delayed hypersensitivity to SRBC than the conventional (CV) mouse. Intravenous sensitisation, which in the CV mouse would give good DH, when elicited in the footpad, did not produce any reaction in the GF mouse, suggesting a relationship to the microbial flora in the intestinal tract.

Inducement via the footpad or other parenteral route of sensitisation did result in some small response following subsequent elicitation within the GF animal, but it was very small compared to what is observed in the conventional. Cell transfers were performed to determine whether this resulted from a T-cell anomaly in the GF animal or whether it was due to some accessory cell defect. In fact, it was discovered that spleen cells from CV mice given to GF mice did not result in a very profound response, suggesting that the defect in the GF mouse is with the accessory cell, the macrophages (*MacDonald* and *Carter*, 1979). This will be discussed in greater detail below in regards to the *Listeria* model. Such studies done in our laboratory and those of others, have shown that GF mice have a lower level of macrophage activation than CV animals, but still demonstrate normal DH responsiveness to contact sensitising agents, while showing no DH to SRBC after i.v. sensitisation, and only limited DH following footpad sensitisation.

Perhaps not surprisingly, we found that conventionalisation or mono-association restored the capacity of the GF mouse to produce a normal DH response to SRBC antigen (*MacDonald* and *Carter*, 1979). In mono-association, the microorganisms with which we had the best results were Gram-negative organisms, a finding which relates then to information discussed below. There was question as to whether there was a B-cell induction, rather than a T-cell induction, of anti-SRBC sensitivity in the GF spleen because of some difference in the processing of antigen in the GF animal. We considered that this might be related to the profound difference in the stimulation of the gut-associated lymphoid tissues. We observed that large germinal centres in the Peyer's patch of a CV animal stained with a monoclonal antibody (Mab) to L3T4, a Mab specific for the helper T-

cell in the mouse. Cells in the T-dependent area in the Peyer's Patches are labelled. The Peyer's patches in the GF animal, because of the lack of the antigenic stimulation by the large numbers of microorganisms ordinarily present in the lumen of the intestine, rarely show germinal centres, characteristic of B-cell and antibody responses, while the T-cell areas are well populated. Upon association with Gram-negative microorganisms, we see an anti-SRBC response similar to what is observed in CV animals and histologic sections of the Peyer's patches would show larger germinal centres. The association of Gram-negative microorganisms in the mammalian intestinal tract with the host's subsequent development of DH and their possible involvement in host immunity to infectious agents was of great interest to us and continues to be.

Related to the above observations are our studies with oral association of mice with *Listeria rnonocytogenes*, a facultative intracellular microorganism which induces a delayed hypersensitivity of some significance (*Czupryuski* and *Balish*, 1981; *Fleming* et al., 1985; *Gray* and *Killinger*, 1966; *Julianelle*, 1942; *Murray*, *Webb* and *Swann*, 1926). In the oral *Listeria* model, we see significant footpad swelling, characteristic of DH to *Listeria* antigens, at day 6; this occurs slightly later, by 2-3 days, than when it would be observed following i.v. infection and probably relates to the fact that DH does not appear until the oral infection results in a systemic infection, involving the spleen. Such a pathogenesis probably accounts for the delay in exhibition of DH and has additional significance. Interestingly, the clearance of *Listeria* from the blood and uptake by the liver and the spleen in the GF and CV animal is exactly the same. In fact, the LD_{50} varies little between the GF or CV mouse. The growth curves of *Listeria monocytogenes* following an i.v. infection in the CV and GF mouse differ slightly but follow the same pattern. The colonisation of the intestinal tract following i.v. challenge as well as oral infection was observed in the GF mouse. The population of *Listeria* achieves high levels and is secondary to the infection of the liver following IV challenge. These organisms, since there is no competition for colonisation sites in the GF animal by *Listeria*, are able to maintain very high levels in the gnotobiotic animal, in this case, mono-associated by *Listeria*, and there is no fluctuation in their populations with time. In the CV animal, *Listeria*, following oral challenge with $5x10^6$, disappears very quickly from the intestinal lumen and is not be detectable after a few days. One of the things which we found to be unusual in our study, and which relates to what was said earlier using inanimate antigens such as SRBC, is a failure of lymphocytes from immunised gnotobiotic mice to transfer resistance to that same microorganism to CV mice. In fact, although the gnotobiotic mouse infected with *Listeria* develops a normal immune response, as far as can be determined, and is resistant to challenge, we were very surprised to find that it was impossible to protect CV recipients with spleen cells from these animals. Although gnotobiotic donors were fully immune to challenge, they were unable to transfer any immunity to the CV animal. In studying this result, we formed the hypothesis that effector cells [sensitised T-cells (*Lane* and *Unanue*, 1972)] leave the spleen and localise in the gut of *Listeria monocytogenes* mono-associated mice, drawn there by the high numbers of *Listeria* in the intestinal lumen of the mono-associated mouse. We would have the reverse effect of what is seen in tuberculosis patients or TB-infected animals late in disease. Early in tuberculosis, it is easy to demonstrate

delayed hypersensitivity in the periphery, i.e., the skin. However, late in the infection, this hypersensitivity is often undetectable and it is thought that the sensitised lymphocytes, which are needed to elicit that peripheral reaction, are all localised in the lung where the severe pathology is occurring thus resulting the peripheral anergy. In the *Listeria* model, our hypothesis is that the reverse is occurring, that is to say, the *Listeria* is able to maintain such high populations levels in the gnotobiotic intestine as opposed to the CV animal, and the antigen load is so great in the gnotobiote, that the sensitised T-cells are localising in the intestine, the site of maximal antigenic stimulation. And thus, we were unable to have significant numbers of sensitised T-cells left in the spleen to transfer this immunity. Such is the basis for our hypothesis. We have considered two ways in which this can be tested: One way, of course, is to remove the T-cells from the intestinal mucosa of these animals and transfer them to recipients but, as *Hiroshi Kyono* (1980) knows, isolating these T-cells in large numbers from the intestinal tract is a significant problem. What we chose to do is the reverse, that is to remove the antigenic load from the intestine and have a reversion, or remigration, of the sensitised T-cells back to the spleen. The latter is the easier one to test and that is what we did. Following the procedures of *Srivastava* et al. (1976), we used a number of non-absorbable antibiotics to reduce the antigen load in the intestinal tracts of *Listeria*-mono-associated mice. In the normal *Listeria* infection, following oral challenge, the organisms reach high numbers in the caecum and infection of the spleen occurs but in animals given the oral antibiotics, colonisation of the gut is completely inhibited, at least based upon what is could be detected on culture media. The oral antibiotics, being non-absorbable, did not affect the pathogenesis of the systemic infection, showing an infection that was turned over at day 3, indicating a very classical response immune response. Furthermore, when spleen cells were taken from these *Listeria* infected animals, in attempting to transfer passive protection, the same insignificant transfer of protection is observed as compared to CV non-immunised mice. When antibiotics were given to reduce the antigen load in the gut, it was possible then to achieve levels of protection that were almost equivalent to those achieved with spleen cells from the CV donor. This then would support the hypothesis of a redirection of the migration of sensitised T-cells. We were still not quite happy with the level of protection exhibited and thought that perhaps there was more to this problem which related to what had been seen with the deficient anti-SRBC delayed hypersensitivity response. This was continued in a thesis study by *Simkins* (1987). Simkins used a battery of monoclonal antibodies in conjunction with flow cytometry (*Herzenberg* and *Herzenberg*, 1978; *Steinkamp*, 1984) to determine the proportion of different T-cell and macrophage populations in CV mouse spleens and Peyer's patches and the same in the GF animals. A significant change in the proportion of macrophages in the spleens of *Listeria*-infected animals as compared to the normal CV animal, and certainly as compared to the macrophage population in uninfected GF mice, was observed. The other numbers are essentially similar. Following infection, macrophages increased from 5% to 13% of total mononuclear cells. Since that cell population changed the most of any that were studied, Simkins suggested (*Simkins*, 1987) that perhaps macrophages were involved in the inability to transfer resistance to *Listeria* with spleen cells from mono-associated

mice. Following antibiotic treatment the macrophage population is observed returning to what it was in the normal GF mouse, between 5% and 6%, as opposed to the 13% seen in *Listeria*-infected gnotobiotic mice. The numbers are taken from flow cytometric profiles; the anti-macrophage Mab, M1/70, is used to label spleen cell populations in CV and gnotobiotic *Listeria*-infected animals respectively. In the gnotobiotic animal there is an even more significant increase in the $M1/70^+$ population. With these observations suggesting the existence of suppressor macrophages, we removed the plastic-adherent cells from donor animals, resulting in profound reduction of $M1/70^+$ population. Except in the case of two of the recipient animals, removal of these cells resulted in a very impressive transfer of protection, well exceeding two logs.

One of the things that strikes us, tying this all together, is that in the *Listeria*-mono-associated animal there is no evidence of germinal centres in the Peyer's patches; this probably relates to the much lower antibody response to *Listeria* infections generally, which is why it is possible to show a good DH response. It is interesting that, unlike mono-association with the Gram-negative microorganisms, we do not see germinal centre formation. What remains to be shown is the exact relationship of the Gram-negative intestinal flora to the general expansion of B-cell populations and the production of antibodies, not only in the local, mucosal lymphoid tissues, but also in the systemic lymphoid tissues. Whether the effect of these microorganisms, and the endotoxin produced by them, is related, in the broader sense, to the development of the capacity for the exhibition of DH remains moot. Apparently, such responsiveness is not induced by association with Gram-positive microorganisms, even though these microorganisms produce a significant amount of peptidoglycan, which has an immune effect similar to endotoxin, as will be discussed by Dr. John Schwab.

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