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How the thymus shaped immunology and beyond

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How the thymus shaped immunology

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Thymectomy, thymus, lymphocyte, graft, tolerance

Abstract

Barely 60 years ago the thymus was considered to be a vestigial organ with no known function or just a graveyard for dying lymphocytes. Now the thymus and its cells cover a vast area of immunology, genetics and epigenetics relating to medicine, including inflammation, infection, vaccination, dysbiosis, immunodeficiency, allergy, autoimmunity, transplantation, tissue repair, pregnancy and cancer. New technology and approaches, now becoming available, will lead to a much deeper understanding of many of these conditions. Hence the thymus and its cells will be occupying researchers and clinicians for decades to come.

Introduction

For centuries the thymus has remained a mysterious organ and contentions and controversies have involved most of the questions regarding its physiology, pathology and clinical significance. There have been many claims that it was an endocrine gland, from whence the name “thymus gland”, but even

to this day there has been no unequivocal demonstration of a thymus hormone.

In the last sixty years, however, the thymus has changed from being a useless structure with no known function to one of the most important organ in the body. How did all this happen in a such relatively short time? The brief answer is through thymectomy. However, not simply thymectomy of adult mice that had been frequently practised by those working on mouse leukemia without any observable immune dysfunction. It happened after **NEONATAL** thymectomy.

This presentation is a personal recollection that will summarize the events leading to the discovery of thymus function and to the identification of the two principal types of lymphocytes, one arising from the thymus itself (T cells) and the other from the bone marrow (B cells), but largely dependent on T cells for optimal function.

History of discovery of thymus function

In 1958, after my internship at the Royal Prince Alfred Hospital in Sydney, Australia, I got a Fellowship to study cancer research at the Institute for Cancer Research in London, UK. There was no space for me in the laboratories in the city and so they sent me to

a satellite Institute in Chalfont St Giles (Buckinghamshire), named Pollards Wood Research Station (Fig 1), where some of their staff were working. As the place was already overcrowded, I was lucky to get a shed in which to work, and to house my inbred strains of mice in a converted horse stables. I mention this because it had an influence on the results of my work that will become evident below. Initially, I had a supervisor, but he soon left having been offered a better position at the National Institutes for Medical Research in London. I was therefore left without a supervisor but lucky to inherit some of his animal space. I describe below the events which took place at Pollards Wood (Institute for Cancer Research) from 1958 to 1965, in NIH in 1963, and from 1966 in Melbourne, Australia.

For my PhD, I chose to work on mouse lymphocytic leukemia, a cancer which arose in the thymus and spread at around 4 to 6 weeks of age. Rather than work on spontaneous leukemia in AKR or C58 mice, irradiation-induced leukemia in C57BL mice or chemically-induced leukemia in DBA/2 mice, as many others had done and were still doing, I opted to work on a new type of lymphocytic leukemia that had recently been induced by Ludwik Gross in the USA [1]. He had made extracts from leukemic tissues of high leukemic strain AKR mice, filtered those extracts, and injected them into low-leukemic strain C3Hf/Gs mice which then developed leukemia at around 3 to 6 months of age, but only

if the filtrate had been injected immediately after birth. Passaging the filtrate serially through several generations of *newborn* low leukemic strain mice gave a filtrate ("Passage A") which yielded 100 percent leukemia. As with other lymphocytic leukemia, it started in the thymus and then spread.

Gross kindly sent me his C3Hf/Gs mice to help me start my PhD. The obvious thing for me to do was not just to show that Gross's procedure worked for me (which it did), but to thymectomize the neonatally inoculated mice when they had reached adulthood at around 6 weeks of age, and to check for the development of leukemia. None of these mice developed the disease [2]. My next move was to graft a neonatal C3Hf/Gs thymus (I used the axilla as a graft site in those days) immediately after *adult* thymectomy (ATx), to determine whether it restored the potential for leukemia development. It did so in 100% of the mice within several months. But then I did something which changed the course of history [3]. Instead of grafting the neonatal thymus immediately after ATx, I grafted it in several groups of mice at different intervals, some 1 to 2 months, some 3 to 5 months and some as late as 6 months post ATx. 90%, 72% and 55% of the grafted mice developed leukemia, respectively [3]. So clearly the neonatally inoculated virus must have remained latent in the absence of the thymus and was reactivated when its target, the neonatal thymus graft, was available. In fact, it was recoverable from the non-leukemic

tissues of neonatally inoculated and adult thymectomized mice [4]. Because of these findings, the obvious question arose as to whether the virus might have multiplied rapidly in some cells found only in neonatal thymus, then spread to other parts of the body before malignant transformation had occurred in the thymus. If so, when the thymus was resected at 1 month of age, the malignant cells, if already present in situ, would have been removed but not the virus which would already have spread out. There was one clear way to prove or disprove this hypothesis. Take out the thymus before injecting the virus, but since Gross's extensive work had shown that the virus must be given at birth, I should perform a *neonatal thymectomy* (Ntx) first and then inject the virus. Subsequently, I should graft a neonatal thymus at different points in time, as done above after ATx (3), and check for leukemogenesis.

The NTx mice, whether inoculated with virus or not, fared well for about 6 weeks. After that some looked sick and wasted [5-6]. Such ill health had never been seen or documented after *adult* thymectomy. The sham-thymectomized (STx) controls held in the same cages as the NTx mice grew perfectly normally. Autopsies showed that the NTx mice had lesions in the liver resembling those induced by the mouse hepatitis virus, as well as a marked depletion of lymphocytes in blood and lymphoid tissues. Since circulating small lymphocytes had some years back been shown

by others to respond immunologically, I grafted the NTx mice with skin from donors of different strains and the results were spectacular, a real eureka moment. Even grafts from strains differing at the MHC (in those days known as H-2) and from rats grew luxuriant tufts of hair [5-7]. Some NTx mice had even been grafted in infancy [8], hence well before the onset of ill health or wasting.

Other investigators reported results of their experiments performed on animals thymectomized at or after birth. Fichtelius *et al* (9) performed *subtotal* thymectomy and sham-thymectomy in *adult* guinea pigs given *Salmonella typhi* H antigen and reported that the antibody titres “tended to be lower” in the thymectomized group. There was no difference between the two groups after a secondary challenge with the same antigen. Archer and Pierce (10) reported, in an abstract in which no data were given, that thymectomy of rabbits performed during the first week of life was followed by subsequent failure to produce antibody to bovine serum albumin.

Many questions and obvious experiments followed and they are listed here.

1. Would thymus grafts restore immune competence? They did so, but the crucial finding was that *foreign* thymus grafts did restore

competence, though not to skin from the same donor as the thymus graft, even if that donor was H-2 disparate. That skin was never rejected [6]. The foreign thymus must thus have induced tolerance to itself and by implication the thymus must have the ability to induce *self-tolerance*. My own words in the paper in which these published data appeared were: “Antigenic material might make contact with certain cell types differentiating in the thymus and in some ways prevent these cells from maturing to a stage where they would be capable of reacting immunologically” and I used the words “selective immunological thymectomy” [6] to describe this negative selection.

2. Since it had previously been shown or believed that the same small lymphocyte was able to initiate both a cellular and a humoral immune response, I checked my NTx mice for their ability to produce antibody. They did so to some antigens, but weakly to others [6, 11].

3. It was essential to show that some lymphocytes must leave the thymus to populate the lymphoid system. However, with no known CD markers and no flow cytometry at the time, I could only do this using thymus grafts and a strain of mice that happened to have a chromosome marker (T6). Thymuses from a C3H newborn mouse were grafted into 7-day old NTx (AKxT6)F1 mice which were immunized with foreign skin 2 to 4 months later. Up to 15 percent

of the cells in metaphase in spleen were found to lack the T6 marker and hence were thymus graft-derived [6].

4. It was well known that the adult mouse had a lymphocyte circulation that by far exceeded that of the newborn mouse. So, it occurred to me that if thymectomy was performed in adult mice that were then totally irradiated (and depending on the dose of irradiation protected with bone marrow), the recovery of the immune response, known to take around 8 weeks in irradiated euthymic mice, would not occur. Thus I thymectomized CBA mice at 8 weeks of age, irradiated them (some with a high dose, 850 rads) at 10 weeks and immediately gave the heavily irradiated mice syngeneic bone marrow (these mice are herein after named "ATxXBM" mice). They were grafted at 16 weeks with skin from different donors. 70 to 77 percent of the ATxXBM mice failed to reject the grafts [12-14].

5. Leukemia researchers had always performed thymectomy in mice between 2 and 3 months of age and had never noticed any defect in immune function. So what would be the result if thymectomy were done in *unirradiated* mice later in life? Checking the response to sheep erythrocytes, I found no difference between STx and ATx mice when the procedure was performed at 4 months of age, but there were differences later, being marked when ATx and STx were done at 9, 18 and 24 months of

age [15]. The sham-operated mice did show some depression of the response when operated at 18 months, but this was not as severe as the ATx mice.

6. As carcinogenic compounds were under intense investigation by others in the Institute for Cancer Research in London, I wanted to test their effect in NTx mice. I applied 3,4-benzopyrene to young adult mice that had been thymectomized or sham-operated. Papillomas occurred in both sets of mice but reached a larger area in the NTx mice. Most importantly, by 180 days 12 percent of skin tumours in the NTx mice became malignant in contrast to only 4 percent in the STx mice [16]. My words in the conclusion of the paper are: "Interference with the cellular immune mechanism may be necessary, in some case, to allow the full expression of a carcinogenic process" [16].

7. Some parotid tumours occurred spontaneously in NTx mice that had been given the Gross virus at birth. Such tumours were known at the time to be the result of polyoma virus infection, a virus that was endemic in mouse colonies. But together with a group headed by Law, I showed that C7BL mice, a strain normally resistant to the polyoma virus, developed such tumours if NTx [17].

When some of the above results were presented at various meetings, many criticisms were given. The most important one was that my mice, having been raised in converted horse stables, must have been exposed to so many intercurrent infections that the additional trauma of NTx or ATx with irradiation precipitated immunodeficiency. This prompted me to go to the National Institutes of Health in Bethesda in 1963, courtesy of an Eleanor Roosevelt Fellowship, to repeat the entire work in germfree tanks. Germfree C57BL mice were NTx or STx in the tank and grafted with H-2 disparate Balb/c skin. None became sick and none of the thymecomized mice rejected the skin [18]. In a separate germfree tank experiment, it was shown that normal newborn mice injected with spleen cells from H-2 different donors became sick and died from graft-versus-host reactions [18].

Identification of T and B cells

Claman *et al.* [19] showed that irradiated mice given syngeneic bone marrow cells and syngeneic thymus cells could produce more antibody than either cell source alone. As no antibody markers were available at the time, the origin of the antibody-forming cells could not be identified.

Having established that the thymus was essential for the normal development of the immune system, I went on to determine

what quantitative difference existed between the recirculating lymphocyte pool of NTx and STx mice. This was done by cannulating the thoracic duct 5 to 6 weeks after birth and draining the lymphocytes continuously for a period of 48 hours after which no further lymphocytes drained out. The cumulative total number of thoracic duct cells drained reached close to 10^8 cells in mice STx at birth but only slightly more than 10^6 cells in NTx mice [20]. Since I had shown that NTx mice failed to reject foreign grafts and often failed to make antibody, I surmised that the cells making up the difference in the two sets of mice were thymus derived and responsible for both cellular and humoral immune responses, whereas the cells found in the NTx mice were derived from another source.

I designed experiments with my first PhD student, Graham Mitchell, to establish once and for all that thymus derived cells were indeed important for both cellular and humoral immune responses. We used the H-2 disparate strains CBA and C57BL and their F1 hybrid, but for simplicity I shall change the name of these strains to simple letters: A and C (Fig. 2). Thoracic duct lymphocytes from first generation hybrid (A x C) F1 normal adult mice were injected intravenously into adult immunoincompetent ATx X (A x C) F1 mice to restore immunocompetence. These hosts, having been heavily irradiated, had to be protected with a source of stem cells and for this we used bone marrow from one

of the two parental strains, namely C strain. After recovery from irradiation, the ATxXBM mice that had received the thoracic duct lymphocytes, were given sheep erythrocytes intravenously and their spleens removed at the previously known height of the humoral antibody response. A spleen cell suspension was made. The number of antibody forming cells was detected by an established plaque assay, in which spleen cells, sheep erythrocytes and complement were poured onto agar plates, incubated at 37 degrees Celsius and clear plaques resulting from an antibody forming cell lysing the surrounding sheep erythrocytes were counted. As the spleens were obtained from the ATxXBM mice that had had their immunocompetence restored by the manipulations described above, a normal antibody plaque forming cell response was obtained as expected. But now came the crucial step. If an anti-A antibody, made by immunizing C mice with A tissues, were added to the plates and lysed all antibody forming cells, no plaques would be detected. That would prove that the (AXC)F1 thoracic duct lymphocytes, (which were known to be mostly thymus derived and had already been shown to be involved in cellular immunity) were responsible for humoral immunity to sheep erythrocytes. The results were spectacularly clear every time the experiments were done and repeated. Adding anti-C antibody to the plates lysed close to 100 percent (86-96%) of plaque-forming cells as expected (both thoracic duct lymphocytes and bone marrow donors have

the C histocompatibility antigens), but adding anti-A antibody lysed 0-12 percent [21, 22].

These results proved beyond any doubt that thymus-derived cells (later called T cells and accounting for more than 90% of the thoracic duct cells found in mice) were not antibody-forming cell precursors. Why then did NTx mice fail to respond to some antigens? Graham Mitchell and I postulated several possibilities, one of which was that antibody forming cell precursors (later known as B cells) could only respond to those antigens if they made contact or collaborated in some way with T cells, as a result of which they received from the T cell a factor (I called it a pharmacological factor) which somehow turned on antibody production.

How did the immunological community react to these findings? Nobel Laureate Burnet expressed reservations “about the significance of results obtained in such biological monstrosities as pure line mice thymectomized, lethally irradiated, and salvaged by injection of bone marrow from another mouse” [23]. Gowans argued that small lymphocytes were morphologically identical and that two rare clonally individuated cells would never find each other to interact at close range: “If we have two cell lines that are collaborating, then we have specificity residing in two cell lines, one thymus-derived and the

other marrow-derived. The problem is to bring these two specific cell lines together. Does this necessity for the two cells to find one another raise problems? It seems an inefficient mechanism if it rests only on chance contacts” [24]. The then Professor of Immunology at the John Curtin School of Medicine in Canberra was less diplomatic and stated that B and T cells represented only the first and last letter of the word bullshit.

Soon, almost every immunologist joined the band wagon and helped work out the intricate details describing cell types, pathways, factors, molecules, etc. Immunology exploded!

Conclusion

The thymus and T cells have pervaded a majority of immunology articles published over the last 40-50 years. T cells appear to be involved essentially across the entire spectrum of tissue physiology and pathology, not just in reactions or diseases considered to be *bona fide* immunological, but also, to cite just some examples, in tissue repair [25], in dysbiosis [26], in pregnancy [27], and in cancer [28]. That vestigial, apparently useless, organ, populated with cells which in 1963 were considered by Nobel Laureate Medawar “as an evolutionary accident of no very great significance” [29], has certainly come of age!

Conflict of interest

None.

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Figure Legend 1: Pollards Wood Research Station,
Nightingales Lane, Chalfont St Giles, Buckinghamshire,
UK, a satellite of the Institute of Cancer Research,
London, UK.

Figure Legend 2: Schematic representation of experiment that
proved the existence of T and B cells. For details see
text.

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