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THE EFFECTS OF ANTIGEN ON THE MIGRATION OF RECIRCULATING LYMPHOCYTES THROUGH SINGLE LYMPH NODES

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Recirculating lymphocytes (RL)[1] are long-lived small lymphocytes which migrate continuously from blood to lymph, predominantly at specialized traffic sites in lymph nodes (LN) (1, 2). In individual LN, recirculation of lymphocytes accounts for over 95% of the cell output (3). In LN undergoing an immune response, where the cell output may increase by as much as 10-fold over the first 48 h after antigen, the migration of RL from blood to lymph still accounts for over 95% of the cell output (3–5).

The observation that increased numbers of intravenous (i.v.)-injected ^{51}Cr-labeled lymphocytes are present in LN after antigen stimulation has been attributed to a failure of RL to leave LN, as a direct consequence of antigen priming (6, 7). The transient fall in cell output in the efferent lymph of an antigen-stimulated LN was originally considered by Hall and Morris as possibly reflecting a temporary reduction in the entry of RL into the LN (8). Hall (9) has since reported that lymphocytes are entering a LN from the bloodstream during a period when the output of cells from an antigen-stimulated LN is decreased.

Thus, at present, the effect of antigen on the migration of RL through LN is interpreted in two contradictory ways. According to one view, antigen provokes an increase in the input of lymphocytes from the blood into LN but is assumed to prevent those same lymphocytes from leaving the LN (6, 7). According to the other view, antigen provokes both an increased input of lymphocytes from blood into LN and an increased output of the same lymphocytes into the efferent lymph (3–5).

Recently, by infusing i.v. ^{51}Cr-labeled autologous RL obtained from efferent lymph draining single lymph nodes, we have demonstrated (10) that the majority of RL required about 30 h to pass from the bloodstream through a single resting LN and reach the efferent lymph. The present experiments were designed to decide between the conflicting opinions on immune response traffic changes and to examine the effects of antigenic stimulation on the migration of RL through single LN. In particular, to determine how the presence of antigen

[1] *Abbreviations used in this paper:* BCG, Bacille Calmette-Guérin; HRBC, horse red blood cells; i.v., intravenous; LN, lymph node; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; PCV, postcapillary venules; PFU, plaque-forming unit; PPD, purified protein derivative; PWM, pokeweed mitogen; RL, recirculating lymphocytes; saline, sterile physiological saline.

affected the input of RL into LN, the transit time of RL through LN, and the output of RL from LN.

Materials and Methods

Animals. Sheep of either sex from commercial flocks of "white alpine" and "black face" aged between 8 mo and 2 yr were used in all experiments. They were kept in metabolism cages and given hay, water, and salt lick ad lib.

Surgery. Lymphatic vessels efferent to the popliteal and prefemoral LN were cannulated using the methods described respectively by Hall and Morris (11) and Hall (12). The lymphatic vessel efferent to the prescapular LN was cannulated by the method used previously (10). Lymphatic vessels afferent to the popliteal node were cannulated by the method described by Hall and Morris (11) and those afferent to the prefemoral LN by the same technique, modified only as the different anatomical location of the prefemoral node required. Animals were allowed 1–2 days (3–4 days when 6–10 ducts were cannulated) to recover from surgery before any experiments were started.

Lymph Collection and Cell Counting. Lymph was collected at room temperature in sterile plastic bottles that were tied to bottle holders sutured to the animal's skin. The collection bottles each contained 1 ml of a solution of sterile physiological saline (saline) containing 500 U USP of heparin (Liquemin, Roche Laboratories, Div. of Hoffmann-La Roche Inc., Nutley, N.J.) and 500 IU each of pencillin and streptomycin (Flow Laboratories, Inc., Rockville, Md.). The collection times were always kept constant at either 3 or 6 h in all experiments involving the collection of ^{51}Cr-labeled RL.

Lymph collection of autologous RL (which were used in all experiments) for ^{51}Cr-labeling was never longer than 16 h. Lymph was usually collected from a prescapular lymph node and only samples free from erythrocyte contamination were labeled. The viability of RL collected in this way was always over 95%, as assessed by trypan blue dye exclusion. Cell counts were made on lymph samples after appropriate dilution using a Coulter counter model Fn with a 100-μm aperture (Coulter Electronics Inc., Hialeah, Fla.). Differential counts were made on Leishman-stained smears.

Labeling of RL with ^{51}Cr In Vitro. RL from efferent lymph were washed twice by centrifugation, incubated for 30 min at 37°C with 30–50 μCi/ml $Na_2{}^{51}CrO_4$ (sp act 245–740 μCi of ^{51}Cr/mg Cr, The Radiochemical Centre, Amersham, England) at a concentration of 10^8 RL/ml of phosphate-buffered saline (PBS) containing 10% lamb serum. After washing three times, the final cell suspension was filtered through a sterile nylon mesh of 30 μm pore size (Nytal, Swiss Silkgauze Factory Co., St. Gallen, Switzerland) to eliminate large cell clumps. This cell suspension was made up to 20 ml with Eagle's minimal essential medium containing 10% lamb serum (ready for i.v. infusion) and two small aliquots taken; one for cell counting and the other for subsequent measurement of radioactivity. The entire procedure gave a cell yield of between 75 and 80%, and the viability assessed by trypan blue exclusion was 85–90%.

Counting of Labeled RL in Efferent Lymph and Individual Lymph Nodes. All efferent lymph samples were treated the same way. After taking an aliquot of 50 μl for cell counting, the lymph was centrifuged at 4°C for 10 min at 1,000 *g*. The cell pellet was then carefully washed with 5 ml of PBS into a polythene tube for later counting in a Packard series 4000 gamma spectrometer (Packard Instrument Co. Inc., Downers Grove, Ill.). Individual LN were surgically removed under anaesthesia, carefully cleaned of all extra capsular fat and connective tissue, weighed, and transferred to polythene tubes for counting in the spectrometer. At the conclusion of a particular experiment all samples were counted at the same time, together with the original aliquot of the infused cells.

Injections. Antigens were usually infused directly into a LN via an indwelling cannula placed in an afferent vessel. Occasionally they were injected subcutaneously in the drainage area of the particular LN. To be certain that the afferent-efferent lymphatic circuit was intact, sterile Evans blue was always included in the solution to be injected.

^{51}Cr-labeled RL were infused over a period of 10 min via a wide bore cannula placed in the jugular vein.

Antigens. Influenza virus; Human influenza virus of the MEL strain (AO/Melbourne/1/35) was

used in a dose of 40,000 hemagglutinating units of virus as determined by the method of Fazekas de St. Groth and Graham (13). This dose amounts to 160 μg of virus, or 4×10^{11} physical particles (14). The stock antigen consisted of a concentrate of purified, noninfective particles suspended in saline, with 0.04% NaN_3 as preservative. *Allogeneic lymphocytes;* RL obtained from efferent lymph from a different breed of animal were exposed to 4,500 rads over 45 min and resuspended in 1 ml of saline. This suspension was then infused immediately after irradiation into the node. *Pokeweed mitogen (PWM);* 15 μg PWM (Grand Island Biological Co., Grand Island, N. Y.) was suspended in 1 ml of saline and injected subcutaneously. *Lipopolysaccharide (LPS);* 200 μg of LPS B (*Salmonella minnesota*, Difco Laboratories, Detroit, Mich.) was suspended in 1 ml of saline and injected subcutaneously. *Bacteriophage ϕX174;* 10^{12} plaque-forming units (PFU) were injected subcutaneously in 1 ml of saline. *Purified protein derivative (PPD);* 100 μg of PPD (lot 280, Ministry of Agriculture, Central Veterinary Laboratory, Weybridge, England) was injected subcutaneously in 1 ml of saline in animals which had been primed 1–2 mo previously with two to three times the human dose of Bacille Calmette-Guérin (BCG) (BCG sec Berna, Swiss Serum and Vaccine Institute, Berne, Switzerland). *Horse red blood cells (HRBC);* 0.5 ml of packed, fresh HRBC suspended in 1 ml of saline was infused into the node.

Presentation of Data. The data are presented in the form of curves, for the sake of visual clarity. The total number of cells collected over a given period was divided by the number of hours of collection, and that arithmetic mean output was then plotted at the midpoint of the respective collection period. ^{51}Cr-labeled RL are represented as concentrations, by plotting the total cpm/10^7 cells collected over that period.

The input of RL from the blood into the lymph compartment of a LN was measured by counting the radioactivity recovered in a LN after a specified period of time after i.v. infusion of ^{51}Cr-labeled RL. The increased input of ^{51}Cr-labeled RL from the blood into antigen-stimulated nodes has been expressed as the percent increase in the input above the control node, according to the formula $(E - C)/C \times 100$, where E = the counts per minute of radiochromium in the experimental node (antigen stimulated) and C = the counts per minute of radiochromium in the control (resting) node.

An increase or decrease in the output of RL from a LN is always expressed relative to the normal output of RL through the particular LN, as determined by prestimulation collections. The prestimulation output measures the normal rate of recirculation of lymphocytes through a LN.

General Experimental Plan. By infusing antigen via a cannulated afferent lymphatic vessel leading to a LN, we could exercise precise control over the amount of, and the time at which, antigen reached a particular node. By collecting the efferent lymph continuously, we could quantitate the output of all cells from a LN for the duration of the experiment. The output of the contralateral node could be followed simultaneously as a control. Using these techniques, five general types of experiments were performed. First, the traffic changes in efferent lymph from nodes responding to different antigens were measured. Secondly, ^{51}Cr-labeled RL were infused i.v. at various times in relation to an immune response in a single LN (which was being continuously monitored in the efferent lymph), and the lymph nodes subsequently removed to determine the increase in the input of RL from the blood. Thirdly, ^{51}Cr-labeled RL were infused i.v. at various times in relation to an immune response, but instead of removing the LN, the appearance of ^{51}Cr-labeled RL in the efferent lymph was examined. The fourth type of experiment simultaneously examined the changes in the afferent and efferent lymph draining the site of subcutaneous injection of antigen. In the fifth series of experiments, the effect of the decrease in the cell output on cells other than RL was examined.

The effect of antigen on the output of cells from afferent and efferent lymph was studied in seven separate experiments. Altogether, 32 sheep were used in following the traffic of RL in 91 individual LN.

Results

Changes in Lymphocyte Output from LN during the Immune Response. The changes in lymphocyte traffic which characterize the immune response of a LN to antigen are shown in Fig. 1. Two features are immediately apparent in all the curves: first, that there is a considerable increase in the lymphocyte output and,

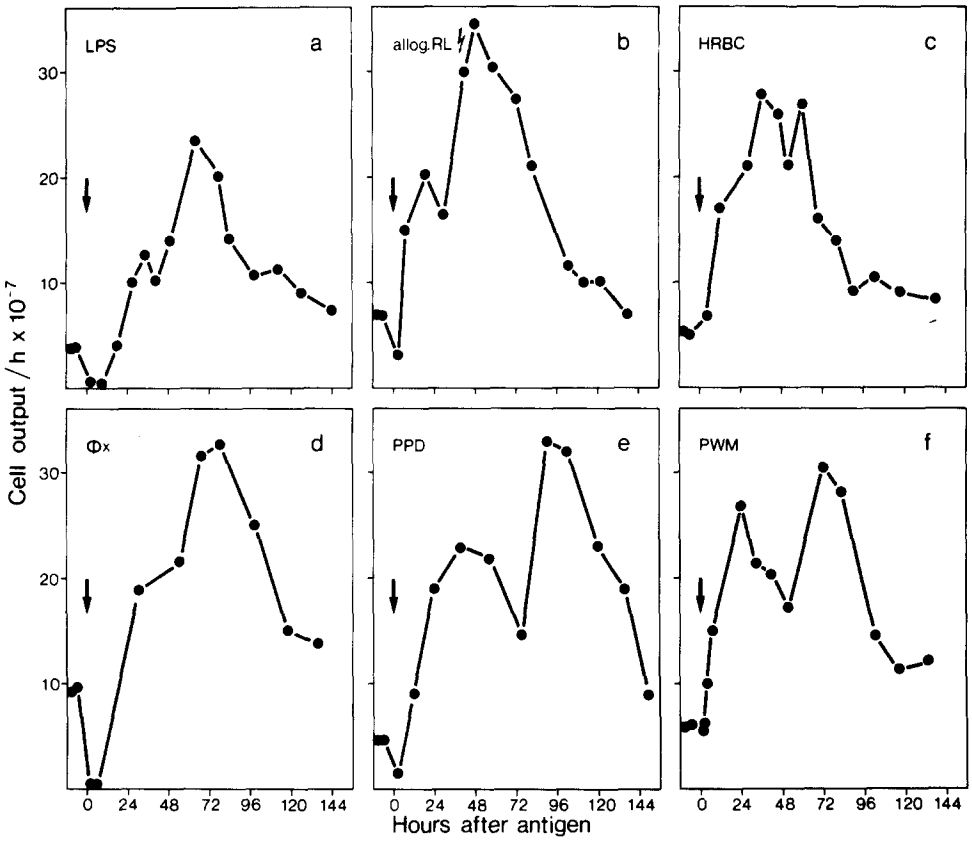


FIG. 1. Each curve represents the total output of cells (●) after stimulation with antigen from the popliteal node of a different sheep. (*a*) LPS, 200 μg injected subcutaneously. (*b*) 4.5 × 10^8 irradiated allogeneic lymphocytes infused over 20 min via a cannulated afferent lymphatic. (*c*) HRBC, 0.5-ml packed cells infused over 20 min via a cannulated afferent lymphatic. (*d*) Bacteriophage φX174, 10^{12} PFU injected subcutaneously. (*e*) PPD, 100 μg injected subcutaneously in a BCG-primed animal. (*f*) PWM, 15 μg injected subcutaneously.

second, that the curves are generally bimodal. In response to some antigens, there is a transient fall in the lymphocyte output immediately after antigen. This fall may be severe and prolonged, as for example with φX and LPS (Figs. 1 *a* and *d*). Here two quite different antigens have rendered the efferent lymph almost acellular for 12 and 18 h, respectively, even though in neither case did the dramatic fall in output herald any substantial anomaly in the pattern of cell output during the ensuing immune response. In the case of the responses to HRBC and PWM (Figs. 1 *c* and *f*), there was virtually no such fall, yet the subsequent immune response traffic changes were effectively the same as those induced by φX and LPS (compare with Figs. 1 *a* and *d*). In the case of allogeneic RL and PPD (Figs. 1 *b* and *e*), a decrease in lymphocyte output of intermediate severity and duration occurred. Over many experiments it has become obvious (Cahill, Frost, Hay, and Trnka, unpublished data) that the decrease in cell output is extremely variable following these antigens and may not occur at all.

Thus, the immune responses shown in Fig. 1 show that the increase in cell output always occurs, while the decrease is variable, does not always occur and is especially severe with a viral antigen.

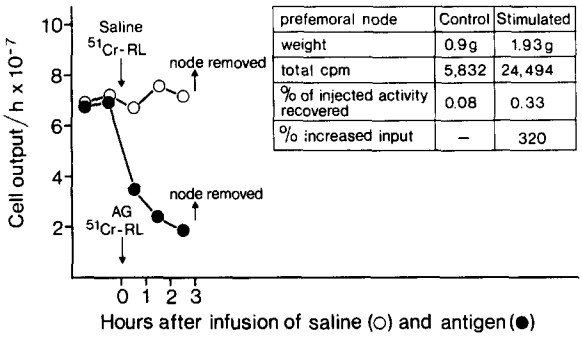
The results presented in Fig. 1 also raise some important questions. What is the cause of the decrease in the lymphocyte output? Is it due to a failure of RL to enter the LN from the blood or is it due to a failure of cells to leave the LN? That is, has the input of RL from blood to lymph at the postcapillary venules (PCV) dropped or has the migration of RL through intranodal pathways been impaired? Furthermore what controls this decrease in cell output and can it occur after administration of antigen at sites other than areas of organized lymphoid tissue; in the periphery for example, where a small scale circulation of lymphocytes is normally occurring? There are additional questions raised with respect to changes in the input of RL at PCV. When do they start in relation to antigen administration? Further, and most importantly, what is their effect on the migration of RL? Do RL move more slowly, are they delayed within the LN as a result of antigen or, indeed, do they move more quickly through the node in view of the enormous increase in the rate of traffic which the antigen-stimulated node has to bear?

Changes in the Input of RL into Antigen-Stimulated LN. In this set of experiments the efferent cell output of the stimulated and contralateral control node was sequentially monitored. One to three pairs of nodes were studied in different experiments. To provoke a highly reproducible and at the same time severe and sustained decrease in cell output, influenza virus was used as the antigen.

^{51}Cr-labeled autologous RL were infused i.v. at the same time as antigen or at various stages during the period of the decrease or increase in the cell output as observed in the efferent lymph; the LN were then removed 3 h later (in one experiment 5 h later) and counted in a gamma spectrometer. Examples of these experiments are given in Figs. 2–4. When ^{51}Cr-labeled RL were infused i.v. at the same time as antigen was infused via an afferent lymphatic into the stimulated node, RL were entering this node in increased numbers, in spite of a 70% drop in cell output, compared to the control node where the cell output was unaltered. More than four times as many ^{51}Cr-labeled RL entered the stimulated node as had entered the control node. There was a 320% increase in the input above the control.

In another experiment, ^{51}Cr-labeled RL were infused i.v. 2 h after antigen was infused into the test node, and the nodes were removed 5 h later. In the interval between i.v. infusion of labeled RL and the removal of the nodes, the cell output from the test node had dropped by 80%. In spite of this striking decrease in the output of RL from the test node, over five times as many ^{51}Cr-labeled RL had entered that node compared with the control node, suggesting that the input of RL into the LN was increasing with time after antigen administration.

The response to ϕX shown in Fig. 1 *d* indicates that the period of decreased RL output has three phases. Phase 1 where the cell output is falling, phase 2 where it remains severely depressed, and phase 3 where it rises again to reach prestimulation levels; phase 3 rapidly rising to a peak in cell output. Fig. 2 shows the entry of cells into lymph nodes during phase 1. The question arose



prefemoral node	Control	Stimulated
weight	0.9g	1.93g
total cpm	5,832	24,494
% of injected activity recovered	0.08	0.33
% increased input	–	320

FIG. 2. The entry of ^{51}Cr-labeled autologous RL into the prefemoral lymph nodes of a sheep. 1.0×10^9 autologous RL labeled with 7.29×10^6 cpm of ^{51}Cr were infused i.v. The output of RL in the efferent lymph draining the left prefemoral node (○) and right prefemoral node (●) is also shown.

whether the input of RL into the LN continued during the later phases of decreased cell output from the LN and, if so, did it change in magnitude during this period. An example of a series of experiments designed to answer this question is shown in Fig. 3. Here the popliteal, prefemoral, and prescapular nodes were all cannulated. Antigen was injected subcutaneously in the drainage area of the right popliteal node 13 h before the i.v. infusion of ^{51}Cr-labeled RL (Fig. 3 *a*), of the right prescapular node 5 h before (Fig. 3 *b*) and of the right prefemoral node at the same time as the infusion i.v. of ^{51}Cr-labeled RL (Fig. 3 *c*). Fig. 3 *c* shows a result similar to Fig. 2, namely that the increased input of RL into LN had started within a short time after antigen administration; in this particular experiment nearly three times as many ^{51}Cr-labeled RL entered the stimulated node as the control. However, when antigen was injected 13 h before the ^{51}Cr-labeled RL were infused i.v. (Fig. 3 *a*), more than seven times as many ^{51}Cr-labeled RL were present in the stimulated node as in its contralateral control (the increase in the input above the control was 611%). Fig. 3 *b* shows that when antigen was administered 5 h before the ^{51}Cr-labeled RL were infused i.v., around four times more ^{51}Cr-labeled RL had entered the stimulated node. These results indicate that the input of RL increases with time after antigen administration.

The high input of RL into the LN continues throughout the immune response. Fig. 4 clearly demonstrates this. The right prefemoral node (Fig. 4 *a*) had received antigen 27 h before the i.v. infusion of ^{51}Cr-labeled RL and the immune response was just at the point where the cell output had reached the prestimulation level when the ^{51}Cr-labeled RL were infused. Over the next 3 h the cell output increased nearly sixfold, indicating the commencement of the peak of increased RL output in the efferent lymph. Over this same 3-h period, nearly twice as many labeled RL had entered the stimulated node as its contralateral control. Similarly, Fig. 4 *b* shows the right popliteal node which received antigen 38 h before the i.v. infusion of ^{51}Cr-labeled RL, and where the peak in the output of RL in efferent lymph was well established, RL were entering the stimulated node at 2.5 times the rate of the control node.

The experiments described so far demonstrate that the increased input of RL

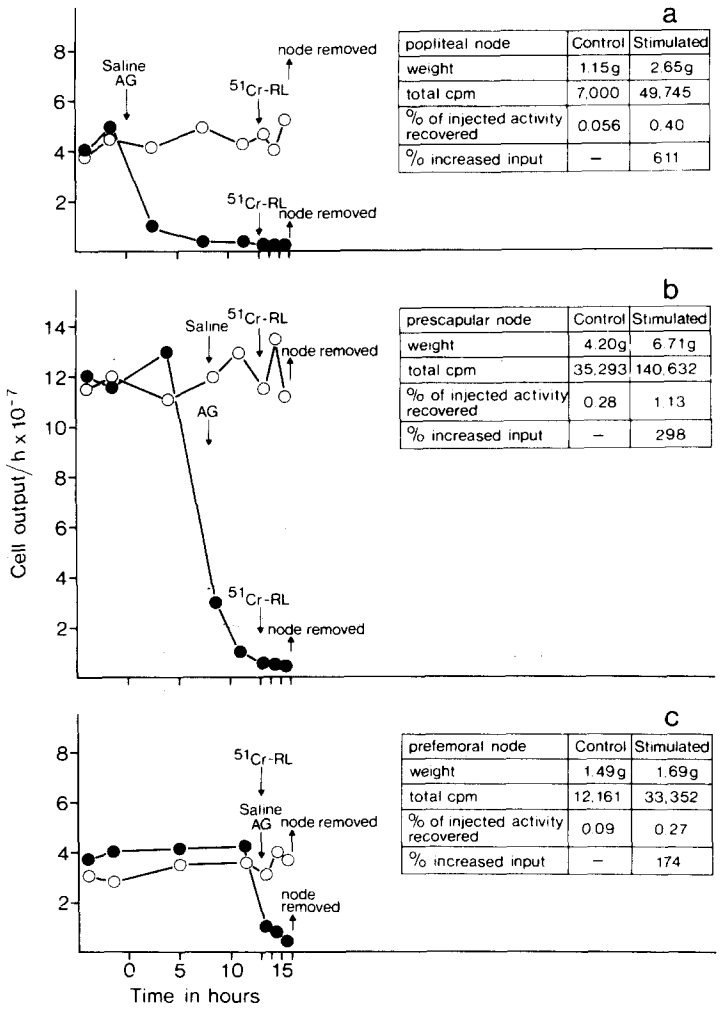


FIG. 3. The entry of ^{51}Cr-labeled autologous RL into the popliteal (*a*) prescapular (*b*) and prefemoral (*c*) nodes of a sheep. 3.91×10^9 autologous RL labeled with 12.50×10^6 cpm ^{51}Cr were infused i.v. The output of RL in the efferent lymph draining the left, control (○) and right, influenza virus-stimulated (●) popliteal, prescapular and prefemoral nodes are shown in Fig. 3 *a*, *b*, and *c*.

into the LN starts very early after antigen entered the LN, but it takes some time to influence the rate of RL output in the efferent lymph. The experiments also show that the increased input of RL into the LN occurs at the same time as their output decreases.

The Transit of RL from Blood to Lymph during an Immune Response. The question to be answered is: Does an antigen, which provokes an increased input of RL into the LN and at the same time a decreased output, alter the kinetics of the recirculation of RL from the blood through that stimulated node? This was examined by studying the appearance of ^{51}Cr-labeled RL in the efferent lymph of antigen-stimulated and resting LN. Two popliteal lymph nodes were compared;

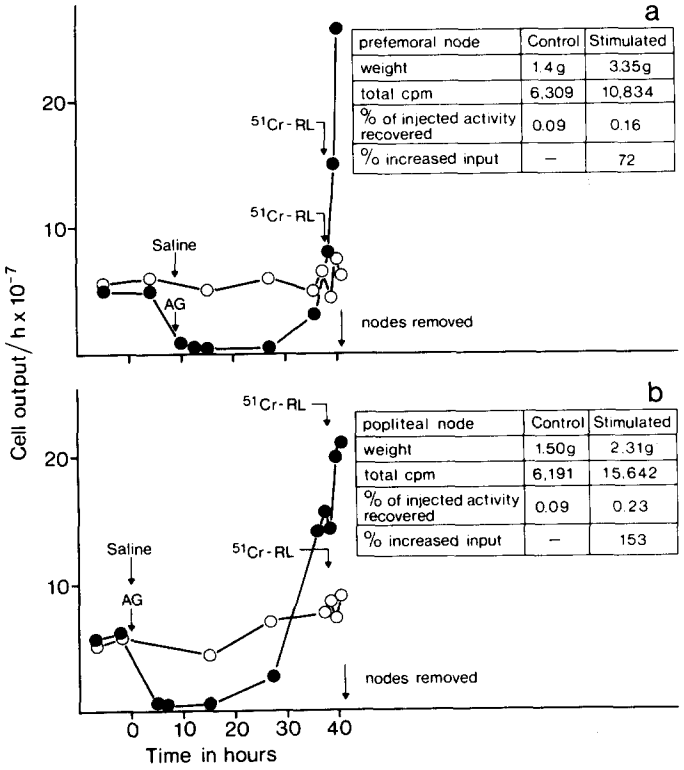


FIG. 4. The entry of ^{51}Cr-labeled autologous RL into the prefemoral (*a*) and popliteal (*b*) lymph nodes of a sheep. 1.32×10^9 autologous RL labeled with 6.51×10^6 cpm ^{51}Cr were infused i.v. The output of lymphocytes in the efferent lymph draining the left, control (○) and right, influenza virus-stimulated (●) prefemoral (*a*) and popliteal (*b*) nodes are shown.

one node was infused with influenza virus via an afferent vessel while the contralateral control node was infused with 1 ml of saline. At the same time ^{51}Cr-labeled RL (collected from the prescapular node) were infused i.v. over 10 min. Fig. 5 *a* shows the enormous increase in cell output after challenge with antigen. The decreased cell output lasted for about 18 h, and for over 12 h the efferent lymph was practically devoid of cells. The two characteristic peaks in cell output are evident and by 72 h the cell output was six times higher than prestimulation levels. At this time the blast cell output (cells produced in the node in response to antigen) was around 50 million cells/h. This was in marked contrast to the control node where the cell output remained constant around 20–30 million cells/h with only a very small percentage (less than 2%) of blast cells.

Fig. 5 *b* shows the appearance of ^{51}Cr-labeled RL in the efferent lymph coming from both nodes. A significant fraction of ^{51}Cr-labeled RL appeared already in the first 3-h collection period. The maximum concentration of ^{51}Cr-labeled RL cells was obtained in both nodes between 27 and 36 h. Clearly, there was no difference in the migration time from blood to lymph of the majority of ^{51}Cr-labeled RL through either a resting LN or a LN undergoing an immune response characterized by a profound decrease followed by a striking increase in

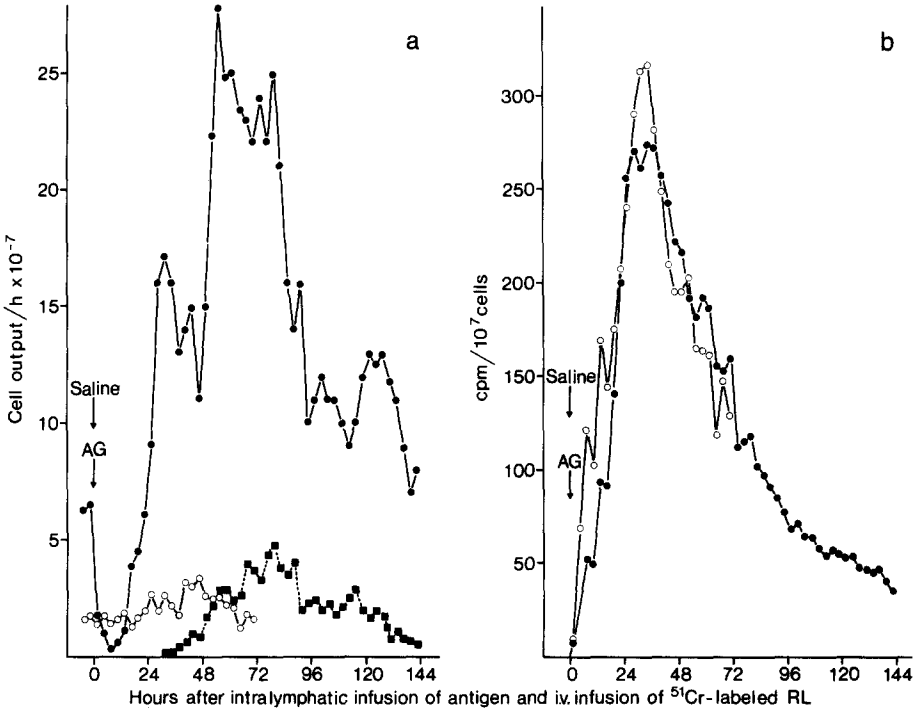


FIG. 5. Fig. 5 *a* shows the cell output (●) and the blast cell output (■) from the right, influenza virus-stimulated and the cell output (○) from the left, control popliteal nodes. Fig. 5 *b* shows the changes in concentration of the ^{51}Cr-labeled autologous RL in the efferent lymph draining the right (●) and the left (○) popliteal nodes. 2.1 × 10^9 autologous RL labeled with 12.1 × 10^6 cpm ^{51}Cr were infused i.v.

RL output. There was, however, a large difference in the absolute number of ^{51}Cr-labeled RL migrating through the antigen-stimulated node compared with the resting node (Table I). Here the absolute number of ^{51}Cr-labeled RL collected in efferent lymph as the immune response progressed is compared with the number collected from the resting node. The results are expressed as total counts per minute or as percent of injected activity recovered. By 75 h (at which time the cannula in the control node ceased to flow), 1.6% of the injected activity had been recovered from the stimulated node compared with only 0.09% from the control node. This represents more than 13 times the number of ^{51}Cr-labeled RL passing through the stimulated than the control node. Indeed, by the end of the immune response 2.1% of the total activity injected was recovered in the efferent lymph draining the stimulated node.

Even though the bulk of ^{51}Cr-labeled RL required the same time to pass through the stimulated and control nodes, in the early samples the concentration of ^{51}Cr-labeled RL in lymph draining the stimulated node was lower than in the control node (Fig. 5). This might suggest that the faster migrating RL were delayed in their passage through the stimulated node. This was not always the case because in other identical experiments, the concentration of ^{51}Cr-labeled

TABLE I
Recovery in Efferent Lymph of ^{51}Cr-Labeled RL after a Single i.v. Infusion

	Time after i.v.* infusion	Total ^{51}Cr-labeled RL recovered	Injected activity recovered
	h	cpm‡	%
Stimulated LN	30	27,058	0.2
	36	52,880	0.42
	75	194,225	1.6
	141	259,665	2.1
Control LN	30	7,885	0.06
	36	12,456	0.09
	75	14,656	0.12
	141§	–	–

* 2.1×10^9 autologous ^{51}Cr-labeled RL with 12.1×10^6 cpm were infused i.v. over 10 min.

‡ cpm represent the total ^{51}Cr-labeled RL recovered in efferent lymph over the time indicated.

§ The control node ceased to flow at 75 h.

RL in the early collections from the stimulated node was slightly higher than that in lymph from the control node.

The experiment presented in Fig. 5 shows the transit kinetics of RL immediately following antigen, i.e. during the period when the increased input of RL into the LN was just beginning. To determine whether there were differences at a later stage, a series of experiments were performed in which ^{51}Cr-labeled RL were infused i.v. much later in the immune response. An example of one of these experiments is given in Fig. 6. Here one popliteal node was infused with influenza virus and the contralateral node was infused with 1 ml of saline. The total cell output curves (Fig. 6 *a*) show that the control node output remained essentially constant while a vigorous immune response followed a period of decreased RL output of more than 18 h in the antigen-stimulated node. ^{51}Cr-labeled RL were infused i.v. 76 h after injection of antigen. At this time the cell output from the node was at its peak for the whole response. ^{51}Cr-labeled RL already appeared in the first 6-h collection and reached maximum concentrations in the period between 24 and 30 h in the lymph draining either the stimulated or control nodes (Fig. 6 *b*). Thus the transit kinetics of ^{51}Cr-labeled RL were the same at all stages of the immune response studied and were not different from those of resting contralateral nodes.

What is the Decrease in RL Output from an Antigen-Stimulated LN due to? In all experiments described so far, ^{51}Cr-labeled RL were infused i.v. either at the same time as antigen was injected into the node or at some time later, and in no case were we able to show any difference between the transit of RL through a resting node and one stimulated by antigen. Yet in all cases antigen had produced a severe decrease in the output of RL in the efferent lymph. This suggested that the decreased output occurred because antigen prevented those RL from leaving which had entered the LN before antigen was given. This

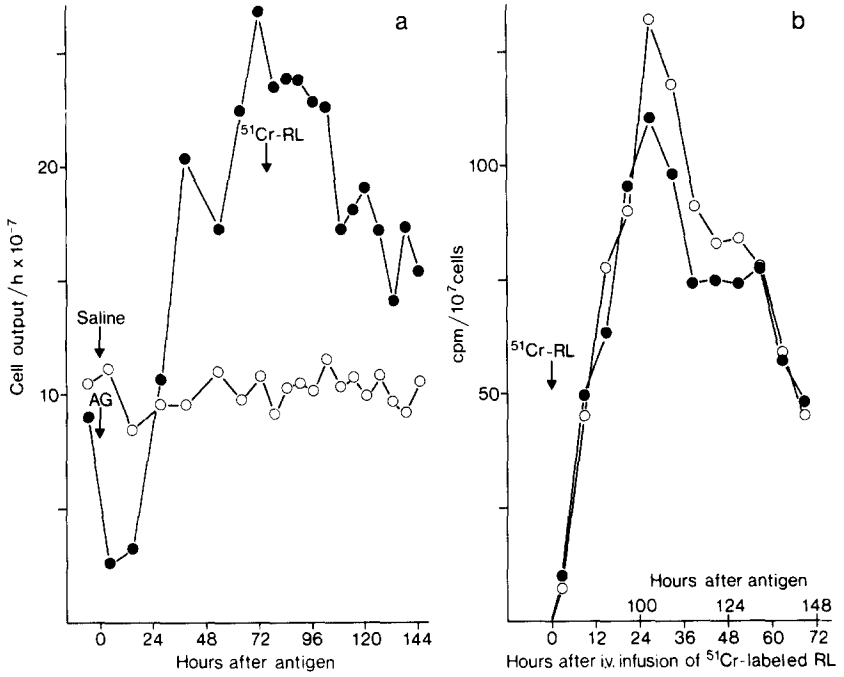


FIG. 6. Fig. 6 *a* shows the cell output from the right, influenza virus-stimulated (●) and the left, control (○) popliteal nodes. Fig. 6 *b* shows the changes in concentration of ^{51}Cr-labeled autologous RL in the efferent lymph draining the right (●) and the left (○) node. 1.6 × 10^9 autologous RL labeled with 4.9 × 10^6 cpm ^{51}Cr were infused i.v. 76 h after injection of saline or influenza virus.

possibility was tested by allowing ^{51}Cr-labeled RL to enter a LN in their normal way (i.e. before antigen induction) and then injecting antigen into the drainage area of the node. In one such experiment two prescapular nodes were compared in the same animal in the following way. One node was given antigen and the contralateral node saline 12 h after ^{51}Cr-labeled RL were infused i.v. The cell output curves (Fig. 7 *a*) show that the output of RL first decreased and then increased in the usual way in the stimulated node. The appearance of ^{51}Cr-labeled RL in the efferent lymph coming from the two nodes is shown in Fig. 7 *b*. In the resting node, ^{51}Cr-labeled RL appeared in the first 6-h collection and reached a maximum concentration over the collection made between 24 and 30 h. The appearance of ^{51}Cr-labeled RL from the antigen-stimulated node, however, was not the same as the contralateral control. The concentration of ^{51}Cr-labeled RL rose in the same way as the control node over that 12-h period before the test node received antigen. When antigen was given, it caused a sharp fall in the output of ^{51}Cr-labeled RL in the efferent lymph of the stimulated node over the period between 12 and 24 h after antigen. This fall in the output of ^{51}Cr-labeled RL coincided with the overall drop in output of RL as shown in the total cell output curves (Fig. 7 *a*). Moreover, ^{51}Cr-labeled RL failed to reach their maximum concentration in the efferent lymph until 36–42 h i.e., 12 h later than in the unstimulated contralateral control node.

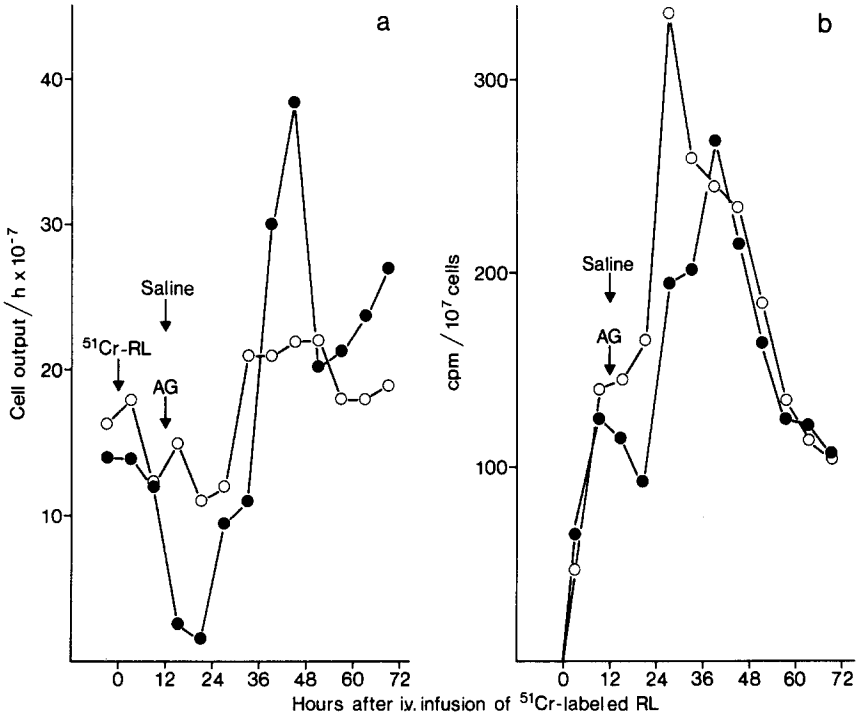


FIG. 7. Fig. 7 *a* shows the cell output from the right, influenza virus-stimulated (●) and the left, control (○) prescapular nodes. Fig. 7 *b* shows the changes in concentration of ^{51}Cr-labeled autologous RL in the efferent lymph draining the right (●) and the left (○) nodes. 7.7×10^9 autologous RL labeled with 13.8×10^6 cpm ^{51}Cr were infused 12 h before the injection of influenza virus.

In a further experiment two popliteal nodes stimulated at different times were compared (Fig. 8). One node received antigen at the same time as ^{51}Cr-labeled RL were infused i.v., while the contralateral node received antigen 12 h later. The cell output curves (Fig. 8 *a*) show that the output of RL from both nodes dropped markedly after antigen was given. The appearance of ^{51}Cr-labeled RL in the efferent lymph draining the two nodes is shown in Fig. 8 *b*. In the node which received antigen first the concentration of ^{51}Cr-labeled RL was low in the first two collections but thereafter it rose rapidly and peaked between 24 and 30 h. Not shown in this diagram is a negative control. This was a prescapular node which received saline at the same time as the i.v. infusion of ^{51}Cr-labeled RL, and here the maximum concentration of ^{51}Cr-labeled RL occurred between 24 and 30 h, too. The second half of the experiment demonstrates just how marked the difference in the appearance of ^{51}Cr-labeled RL is when antigen is given 12 h later. Thus immediately on receiving antigen there is a precipitous fall in the appearance of ^{51}Cr-labeled RL which coincides with the decrease in RL output (see Fig. 8 *a*). Between 24 and 30 h, the concentration of ^{51}Cr-labeled RL was at its lowest level from this node, at precisely the time when it was at its highest in the node which received antigen 12 h earlier. The peak concentration of ^{51}Cr-

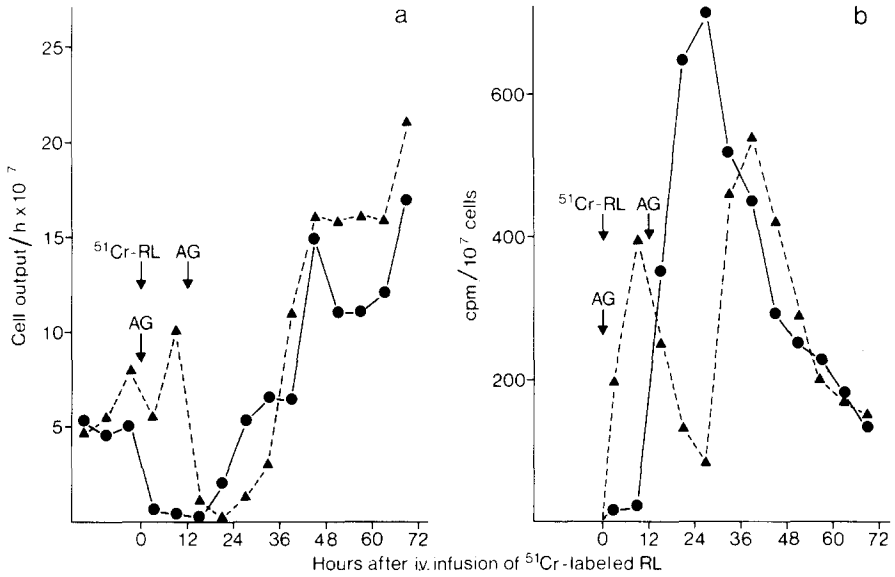


FIG. 8. Fig. 8 *a* shows the cell output from the right popliteal node (▲) which received influenza virus 12 h after the infusion of ^{51}Cr-labeled autologous RL and the left popliteal node (●) which received influenza virus at the same time as the infusion of the labeled RL. 5 × 10^9 autologous RL labeled with 17.2 × 10^6 cpm of ^{51}Cr were infused i.v. Fig. 8 *b* shows the changes in concentration of ^{51}Cr-labeled autologous RL in the efferent lymph draining the right (▲) and the left (●) node.

labeled RL in lymph draining the node which received antigen 12 h later occurred between 36 and 42 h. The experiments illustrated in Figs. 7 and 8 demonstrate that ^{51}Cr-labeled RL which had entered the node before antigen was given were, on administration of antigen, prevented from leaving that node. RL brought into a node by the administration of antigen, on the other hand, migrated through that node in the same way as they did through a nonstimulated node.

The Effect of Antigen on Cell Traffic in Peripheral Lymph. It has already been shown by Smith et al. (15) that only under special conditions, such as during the formation of a peripheral granuloma, does antigen cause an increase in the traffic of lymphocytes in peripheral lymph. It is not known whether antigen will produce a decrease in the output of lymphocytes in peripheral lymph. Fig. 9 shows an experiment designed to answer this question. Antigen was injected subcutaneously into the drainage area of a popliteal lymph node. The consequent changes in cell traffic were then monitored continuously for 150 h in both the efferent lymph and in the afferent lymph, draining the site of injection of antigen. The recovery of the Evans blue (injected with the antigen) in the afferent lymph assured us that the afferent vessel was draining the site of antigen injection. The recovery of dye in the efferent lymph ensured we were not draining away all the antigen in the afferent vessel.

In Fig. 9 *a* the total cell output and the blast cell output from the efferent lymph is contrasted with the changes in the total cell output of the afferent

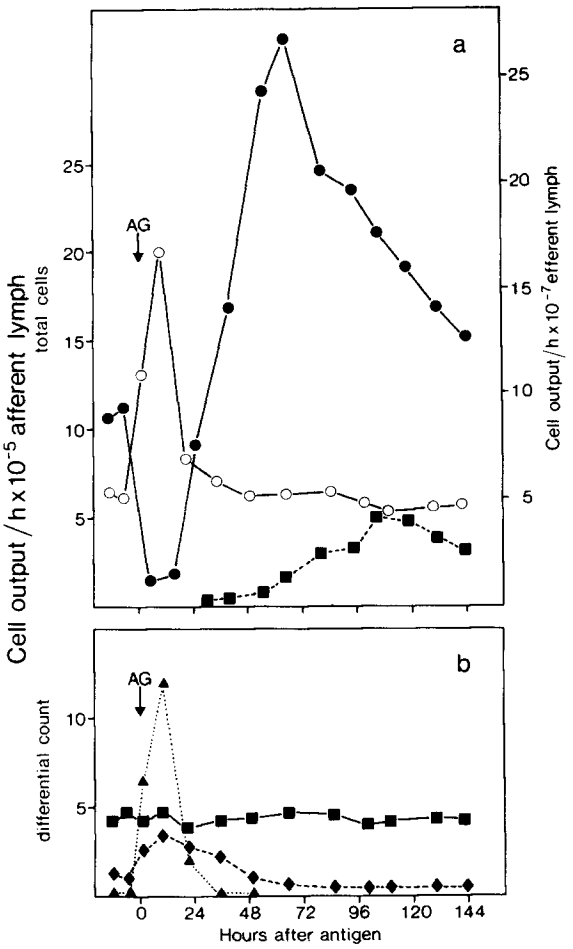


FIG. 9. Simultaneous changes in the cell output from afferent lymph and efferent lymph from the right popliteal node draining the site of injection of influenza virus. Fig. 9 *a* shows the total cell output (●—●), the blast cell output (■- - -■) from the efferent lymph and the total cell output from afferent lymph (○—○). Fig. 9 *b* shows the differential cell output from afferent lymph. Small lymphocytes (■—■), granulocytes (▲- - -▲) and macrophages (◆- - -◆).

lymph. The efferent lymph shows a vigorous immune response to antigen. After an initial decrease in the output of RL the total cell output increased to about three times the prestimulation level and by 100 h the node was producing around 50 million blast cells/h. The changes in cell output of the afferent lymph were quite different. 2 h after antigen the total cell count had doubled and in the collection between 2 and 18 h had increased more than threefold, but in the next collection period between 18 and 28 h it had almost returned to prestimulation levels. Thus, over the period when the total cell output from efferent lymph had fallen by over 95%, that from the afferent lymph had risen by more than 300%.

Fig. 9 *b* shows the changes in the differential cell count which occurred in the

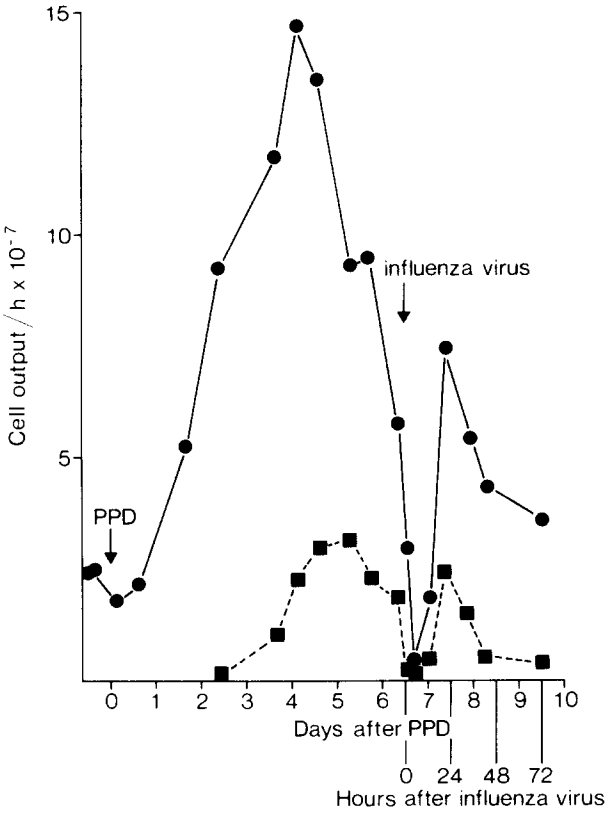


FIG. 10. Cell output from a left popliteal node after the injection of PPD and subsequently injection of influenza virus. The total cell output (●) and the blast cell output (■) are shown.

afferent lymph. The increased output was largely due to the transient appearance of a burst of granulocytes; in this example predominantly neutrophils, although in some experiments large numbers of eosinophils were present. The increase was in part due also to an accumulation of macrophages. Many of these macrophages had the appearance of blood monocytes in contrast to the macrophages present before antigen which had numerous long pseudopodia and many cytoplasmic vacuoles. The morphological scoring of cells as macrophages was supplemented in some experiments by injecting carbon particles together with the antigen or by incubating afferent lymph with carbon particles for 30 min at 37°C. The former method gave the largest number of cells with ingested carbon and confirmed that many of the cells that looked like monocytes were actively phagocytic. It also showed many polymorphs crowded with ingested carbon particles. The in vitro technique gave considerably fewer cells containing carbon particles, but otherwise similar results.

Significantly, the output of small lymphocytes in afferent lymph remained virtually unchanged at about 4×10^5/h over the entire 150-h collection period in complete contrast to efferent lymph where the decrease and subsequent increase in cell output occurred as usual. The fall in lymphocyte output seems to occur therefore only when antigen interacts with cells in organized lymphoid tissue.

The Effect of the Decrease in Cell Output from a LN on Cells other than RL. The decrease in cell output as described so far has been shown to affect RL. The question remained whether other cells would be prevented by antigen from leaving a node, or whether the decreased output was specific for small lymphocytes. This problem was approached by stimulating an immune response to one antigen and then, when the blast cell response to that antigen was well advanced, giving influenza virus and examining consequent cell traffic changes. The first antigen given was PPD (Fig. 10) but in other experiments identical results were obtained when allogeneic lymphocytes were used instead. Fig. 10 shows a vigorous immune response to PPD. About 4 days after giving antigen, the total cell output was almost six times greater than prestimulation levels. Influenza virus was given 156 h after PPD and although the cell traffic changes were declining the blast cell output was still 20 million/h. 9 h after giving influenza virus, the total cell output had fallen by 95% and the blast cell output had fallen to almost zero. Thus the output of blast cells which were produced in the node itself in response to PPD, was decreased in the same way as RL i.e., the drop in cell output was not specific for small lymphocytes.

The changes in the efferent lymph were followed for 74 h after influenza virus had been given which meant for 10 days after PPD was injected. Yet, although a decrease in cell output occurred, there was no subsequent increase in the cell output in response to the influenza virus. When influenza virus was given without prior immunization by another antigen the total cell output had always increased and often reached its maximum level by 74 h. In this experiment the response to PPD appeared to follow its usual course apart from the virus-induced decrease in cell output and when the experiment was terminated after 10 days, the PPD response was nearly over. Thus the second decrease in cell output was not followed by a second increase in cell output.

Discussion

The experiments reported in this paper deal with the interaction of RL and the blood vascular endothelium. They demonstrate that LN control the orderly recirculation of RL both under resting conditions and during an immune response. In all our experiments the animal has served as its own control, and the input and output of its own ^{51}Cr-labeled RL from individual antigen-stimulated LN has been monitored and compared with that from resting contralateral nodes.

Our data show that the input of RL into a LN starts to increase within 3 h of antigen administration, that it increases in intensity to around 13 h and that it continues throughout the immune response, returning to prestimulation levels when the response is over. Thus the increased output of RL, as later observed in the efferent lymph, reflects events which occurred in the LN itself at the very beginning of the immune response.

We have shown previously (10) that the blood concentration of i.v.-infused ^{51}Cr-labeled autologous RL falls by 50% in the first 3 h after infusion. Since labeled RL migrate via PCV into LN at a rate proportional to their concentration in the blood (16), in the present studies the rate of entry of ^{51}Cr-labeled RL into individual LN will be maximum in the first 3 h after infusion. As a result,

the greatest changes in concentration of ^{51}Cr-labeled RL in efferent lymph will be due to those RL which entered over this period. In every experiment where ^{51}Cr-labeled RL were infused i.v. at the time or any time after antigen was administered to a particular LN, the highest concentration of labeled RL in the efferent lymph draining either antigen-stimulated or control nodes was obtained in the same collection periods. We conclude therefore that, at any stage during an immune response, the majority of RL require the same time to pass from blood to lymph through a stimulated LN as they require to pass through a resting LN. Hence the increased output of RL from a LN during an immune response is due principally to an increase in the number of RL passing through a LN. The transit time is age dependent, being shorter in younger animals (10); in most of the experiments described here it was between 24 and 30 h.

We have been able to collect quantitatively all the ^{51}Cr-labeled RL emerging from antigen-stimulated single LN. Other authors, not making use of this facility, observed the increased entry of i.v. injected ^{51}Cr-labeled LN cells into antigen-stimulated LN and concluded that the ^{51}Cr-labeled cells were being trapped with the LN (6, 7, 17–20). In this case the trap would be the LN, trapping ^{51}Cr-labeled LN cells which, being prevented from leaving the LN, would not be expected to appear in efferent lymph. Because our experiments indicate that about 14,000 RL/s are passing from blood to lymph through a single resting popliteal LN (weighing approximately 1 g) and that this can increase to about 140,000 RL/s in response to antigen without any alteration in transit time for the majority of RL, the term lymphocyte trapping seems inappropriate. The trapping of the small minority of specific antigen-reactive cells is another matter altogether (21–24), and even if such specific cells were to make up 10% of the RL pool, methods of the type being discussed would not be sufficiently precise to demonstrate specific trapping.

The data indicate that antigen can cause a delay in the exit through the efferent lymph of cells which were in the lymphoid compartment of a LN before antigen reached it, and that this happens at the same time as the input of RL into the LN is increasing. They further indicate that in the peripheral tissue spaces, antigen administration does not provoke a decrease in the output of lymphocytes from peripheral lymph. Furthermore, the increased input of RL into LN can take place without the decrease in RL output and vice versa. Taken together, these observations suggest that the ability to decrease the output of RL is a property of LN and that it operates independently of the high endothelial PCV. It is interesting to note in this context that Ford (25), using swine influenza virus, was unable to show an effect analogous to the decreased output of RL from LN in the isolated perfused rat spleen. This phenomenon might occur only in LN. Our data do not show how the decrease and increase in the output of RL from LN undergoing an immune response are controlled, although they do define some of the parameters necessary for their occurrence and suggest that they might be controlled by different mechanisms.

Summary

The increased input of RL into LN starts within 3 h after antigenic stimulation and is due principally to an increase in the number of RL passing through a

LN. It is not associated with an altered transit time through a LN of the majority of RL and it cannot occur in the absence of high endothelial PCV. The immediate decrease in the output of RL from an antigen-stimulated LN is due to a delay in the exit from that LN of RL which had entered the LN before antigen was given. The decrease in cell output from a LN after antigen administration affects blast cells produced within the LN as well as small lymphocytes and is not specific for RL.

There are at least two distinct mechanisms controlling the migration of RL through an antigen-stimulated LN. The first controls the increased input of RL which occurs only through high endothelial PCV. The second controls the immediate decrease in cell output, which although it does not occur at the level of the high endothelial PCV, can only occur in organized lymphoid tissue. The increased input of RL into an antigen-stimulated LN and the decreased cell output from the LN can occur independently and are possibly controlled by different mechanisms.

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