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Injectional anthrax infection due to heroin use induces strong immunological memory

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Dear Sir,

There was a cluster of anthrax cases in heroin users in 2009–2010 and again in 2013, contamination with anthrax of Turkish origin causing often fatal disease. We show here that exposure to anthrax in this manner elicits strong T cell immunity, even in the face of extremely severe disease. The pattern of immunity seen is reminiscent of immune responses in hyperimmunized vaccines or naturally exposed farmers. This robust response to anthrax is contrary to expectation when one considers the well-documented ablative effect of anthrax toxins on immune function.

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There has been growing concern about infection by spore-forming bacteria leading to soft-tissue infections and other severe manifestations in heroin users. Clusters of infection derived from contaminated drug batches were seen during 2009–2010 when there were over 80 anthrax cases in Scotland and SE England, seemingly related to a single batch of anthrax-contaminated heroin.[1–3] There have been a further 13 cases in the UK, Germany, Denmark and France during 2012–2013.[4] Contamination most likely occurs through exporting drug batches concealed in animal hides, through the manufacturing laboratory containing equipment contaminated with anthrax spores or through heroin having been 'cut' with contaminated material such as powdered bone. Genotypic analysis of the UK cases confirms that all are likely to derive from a single anthrax strain, closely related to strains from Turkey and compatible with the notion of contamination during export to Europe.[5,6]

The majority of cases have been fatal. Previous experience of anthrax has been, most commonly, through inhalational contact in 'wool-sorters' disease,[7] cutaneous exposure in tanners and goat-herders, as well as ingestional exposure through eating infecting livestock. More rarely, there have been instances of deliberate inhalational anthrax exposure, most notoriously the cases following the attacks through the US postal system.[8] There are no previous reports, outside of animal model experiments, of exposure to anthrax spores through direct injection or by subcutaneous injection ('skin-popping'). These modes of infection are associated with particularly severe and diverse pathological outcomes.

Understanding the bacterial pathogenesis and immunology of anthrax infection is important if we are to develop better strategies for supporting the septic patient exposed to injectional anthrax. Experimental models suggest that the anthrax toxins that target immune cells ablate adaptive immunity to the bacterium.[9] We show here however, in detailed analysis of T cell immunity in a survivor of injectional anthrax, that strong immunity can be developed, reminiscent of that seen in vaccinated individuals.[10]

We describe here a 60-year old man who presented to a West London hospital with a fever and left femoral artery pseudoaneurysm one day after injecting heroin into the left groin. There was a pulsatile mass in the left groin; at surgery for the pseudoaneurysm repair, necrotic tissue was debrided. On admission, broad spectrum intravenous antibiotics were started and continued for ten days. These included Ciprofloxacin 500 mg PO BD, Clindamycin 600 mg IV QDS, Flucloxacillin 2 g IV QDS, Benzylpenicillin 2.4 g IV 4 hourly and Metronidazole 500 mg IV TDS. At day 19, further debridement was required and he was recommenced on broad spectrum antibiotics for a further 14 days. Tissue samples taken at this debridement were negative for anthrax on culture and PCR. Serology was subsequently received which was strongly positive. Blood samples were obtained at 5 months after discharge with full, informed consent; ethical approval was obtained under LREC 11/H0721/15. Peripheral blood mononuclear cells (PBMC) were separated by Ficoll Paque centrifugation and CD4 T interferon (IFNgamma) ELIspot responses assessed by culture with overlapping synthetic 20-mer peptides representing the amino acid sequence of anthrax protective antigen (PA) and lethal factor (LF),[10] final concentration of 25 μg ml.

We have previously conducted an extensive analysis of the immunogenicity of epitopes from anthrax PA and LF toxins in agricultural workers from the Kayseri region of Turkey

who had been hospitalized following occupational exposure to cutaneous anthrax.[10–12] In that study we found that, despite the extensive literature on the immunotoxic impacts of anthrax, survivors had strong adaptive immune memory, mapping to a number of immunodominant CD4 epitopes. This is likely the reason that reinfection is never seen in those communities. Furthermore, while most efforts regarding protective vaccines have targeted PA, one of the other toxins, LF, was at least as immunogenic in the context of natural infection. Considering the differences between cutaneous anthrax and the very severe clinical picture produced by injectional anthrax, we questioned whether T cell memory had been established in this individual. We found strong CD4 T cell IFNγ responses to epitopes within 4 of the 20-mer peptides in our PA peptide library and 4 of the peptides in our LF library (Table 1). No responses to these peptides were found in unexposed, healthy control donors.[10] We related this immune response to other datasets in our lab: PA and LF T cell responses of naturally exposed agricultural workers in Turkey, PA and LF T cell responses of defense workers hyperimmunized with the anthrax vaccine precipitated (AVP) vaccine. We also considered the binding affinity of the anthrax T cell epitopes to common HLA class II alleles in the population, a means of identifying those responses that are of the broadest applicability.

In this individual 4 epitopes from PA elicited strong CD4 T cell responses, PA 41–60, 181–200, 451–470 and 661–680. All of these except 181–200 have previously been seen by us as strong immune epitopes in mapping T cell immunity in naturally exposed Turkish farmers. We found that PA 41–60 was also strongly immunogenic in HLA class II transgenic mouse strains carrying each of 2 common alleles, HLA-DR1 and HLA-DQ8 (data not shown). PA 181–200 was immunogenic in HLA-DR15 transgenics and PA 661–680 in HLA-DR4 transgenics. In HLA class II peptide binding studies, this latter peptide had the property of binding most alleles tested.

The four epitopes recognized within the LF sequence were LF 41–60, 71–90, 287–306 and 507–526. LF 41–60, 71–90 and 287–306 have previously been noted by us as highly immunogenic in the Turkish cohort. LF 41–60 and 287–306 are also recognized by T cells in AVP vaccinees.

Analysis of adaptive immunity to *Bacillus anthracis* is of particular interest from the perspective of pathogenesis. However, one might predict that this might be confounded by the mode of action of anthrax toxins: a substantial literature addresses the effects of anthrax toxins on ablation of immune signaling and activation at several different levels – antigen presenting cell function, B cell and T cell signaling.[9,12] Nevertheless, the patient described here developed strong, potentially protective T cell immunity to several commonly immuno-dominant epitopes of PA and LF. This response is similar to that of hyper-immunized vaccines or agricultural workers who have recovered from cutaneous anthrax. In experimental models, immunization route can have profound impact on qualitative and quantitative aspects of T cell immunity, including patterns of epitope recognition. For anthrax exposure, injectional exposure seems to give similar results to intramuscular vaccination or cutaneous contact with animal hides.

An improved understanding of adaptive immunity to anthrax in these unusual patients has value in consideration of which may be the most immunogenic components of the bacterium for targeting in next-generation vaccines.

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Table 1

Summary of identified anthrax Protective Antigen (PA) and Lethal Factor (LF) CD4 T cell epitopes. PBMC were cultured in IFNγ ELIspot plates (Diaclone, UK) for 72 h in the presence of 20-mer peptides overlapping by 10 from the amino acid sequence of *Bacillus anthracis* PA and LF. Results were expressed as delta spot forming cells per 10^6 cells (ΔSFC/10^6) (SFC/10^6 of stimulated cells minus SFC/10^6 of negative control). Peptides were considered positive if the ΔSFC/10^6 was more than two standard deviations above the negative control (patient cells plus medium) mean value and ≥50 spots. A peptide was only considered to contain an epitope if both pools in which it occurred were positive. This resulted in the identification of 4 epitopes for PA and 4 epitopes for LF. The epitopes are shown with related data. A) T cell ELIspot responses to anthrax LF epitopes, B) Relative binding affinities of these peptides to common HLA-DR alleles, C) T cell ELIspot responses to anthrax PA epitopes, D) Relative binding affinities of these peptides to common HLA-DR alleles.

A

CD4 T cell epitope, lethal factor (LF)	IFNγ response ΔSFC/10^6	Evidence from other cohorts of CD4 T cell response to this epitope
^{41}GMHVKEKEKNKDENKRKDEE^{60}	138	Natural exposure to cutaneous anthrax (Kayseri patients); anthrax vaccine precipitated (AVP) vaccinees
^{71}EIMKHIVKIEVKGEEAVKKE^{90}	106	Natural exposure to cutaneous anthrax
^{287}LSLEELKDQRMLSRYEKWEK^{306}	136	Natural exposure to cutaneous anthrax (Kayseri patients); anthrax vaccine precipitated (AVP) vaccinees
^{507}NFKYSISSNYMIVDINERPA^{526}	71	–

B

CD4 T cell epitope, lethal factor (LF)	Relative binding affinity of peptide to HLA-DR molecules[a]						
	DR1	DR3	DR4	DR7	DR11	DR13	DR1501
	14%	30%	20%	25%	14%	16%	27%
^{41}GMHVKEKEKNKDENKRKDEE^{60}	>2563	>1000	>1250	>3365	>1357	78	>208
^{71}EIMKHIVKIEVKGEEAVKKE^{90}	243	50	567	249	4	19	329
^{287}LSLEELKDQRMLSRYEKWEK^{306}	31	3	>1250	1414	17	3	18
^{507}NFKYSISSNYMIVDINERPA^{526}	0.3	ND	0.2	1	10	118	44

C

CD4 T cell epitope, protective antigen (PA)	IFNγ response ΔSFC/10^6	Evidence from other cohorts of CD4 T cell response to this epitope
^{41}GDLSIPSSELENIPSENQYF^{60}	368	Natural exposure to cutaneous anthrax (Kayseri patients)

A		
^{181}DGIPDSLEVEGYTVDVKNKR^{200}	143	–
^{451}DTDQVYGNIATYNFENGRVR^{470}	383	Natural exposure to cutaneous anthrax (Kayseri patients)
^{661}DMLNISSLRQDGKTFIDFKK^{680}	15	Natural exposure to cutaneous anthrax (Kayseri patients)

D

CD4 T cell epitope, protective antigen (PA)	Relative binding affinity of peptide to HLA-DR molecules[a]						
	DR1	DR3	DR4	DR7	DR11	DR13	DR1501
	14%	30%	20%	25%	14%	16%	27%
^{41}GDLSIPSSELENIPSENQYF^{60}	3312	>728	46	424	>1288	>1288	134
^{181}DGIPDSLEVEGYTVDVKNKR^{200}	>6667	20	>1788	>1543	>1336	>1908	134
^{451}DTDQVYGNIATYNFENGRVR^{470}	200	>667	293	30	>1336	>1908	2
^{661}DMLNISSLRQDGKTFIDFKK^{680}	200	2	30	27	33	4	27

[a]Results are expressed as a relative binding ratio obtained by dividing the IC50 of peptide by that of a reference peptide that binds strongly to the HLA molecule. Lower numbers correspond to a higher binding affinity. Numbers in bold (ratio 20 or less) = high affinity binding; numbers in bold (ratio 20–100) = moderate binding affinity. Each peptide – MHC combination was evaluated in 3 independent experiments. Percentages are the frequency of the HLA-DR alleles in a Caucasian population calculated from http://www.allelefrequencies.net Allele frequency net: a database and online repository for immune gene frequencies in worldwide populations. (Ref. Gonzalez-Galarza FF, Christmas S, Middleton D and Jones AR *Nucleic Acid Research 2011, 39, D913–D919*).