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Immunohistochemistry analysis of pulmonary infiltrates in necropsy samples of children with non-pandemic lethal respiratory infections (RSV; ADV; PIV1; PIV2; PIV3; FLU A; FLU B)

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ABSTRACT

Background: Acute viral respiratory infections represent a globally important cause of morbidity and mortality in childhood. An individual's cellular response appears to play a critical role in recovery from infections, given that individuals with impaired cellular immunity, congenital or acquired, have more severe diseases and secrete the virus for longer periods.
Objectives: The aim of this study was to immunohistochemically evaluate the expression of the cell surface antigens CD4, CD8, CD25, CD14 and CD74, in pneumonic infiltrates in the alveolar septa using paraffin-embedded lung samples from autopsies of immunocompetent children who died of lethal, non-pandemic, severe acute respiratory infections.
Study design: From 794 cases of pediatric autopsies of patients with severe respiratory disease (between 1960 and 2004), 193 cases were selected for this study. To identify subpopulations of inflammatory cells in the alveolar septa, cell surface antigen expression was assessed by immunohistochemistry using the following primary antibodies: anti-CD4, anti-CD8, anti-CD14, anti-CD25 and anti-CD74.
Results: The TCD8+ lymphocyte count was higher in the virus-positive group ($p = 0.04$) and was also much higher among cases that were positive for more than three viral types ($p = 0.016$). There were fewer CD14+ cells in cases of AdV (adenovirus) infection ($p = 0.002$), and there was a predominance of CD74+ cells in the histopathological pattern defined as interstitial pneumonitis ($p = 0.037$).
Conclusions: The results of this study demonstrate that TCD8+ lymphocytes present in the alveolar septa participate to a greater extent in the response toward viral pneumonia, while CD14+ cell numbers are often reduced in cases of AdV.

1. Background

Acute respiratory infections are a major worldwide cause of morbidity and mortality in childhood, particularly in developing countries. The data from the World Health Organization (WHO) have shown that, in the last decade, approximately one-third of the global mortality in children, accounting for 4–5 million annual deaths, was caused by acute respiratory infections [1,2].

The most common viruses causing viral pneumonia are influenza virus (FLU A and FLU B), parainfluenza (PIV1, PIV2, PIV3), adenovirus (AdV) and respiratory syncytial virus (RSV) [3]. In the respiratory tract, humoral immunity against influenza viruses involves neutralizing antibodies (immunoglobulin A, lymphocyte B and plasma cells) [4–6].

With respect to the cellular immune response, TCD4+ lymphocytes attract immune cells in response to major histocompatibility (MHC) class II antigen presentation, while TCD8+ (cytotoxic) lymphocytes induce the MHC class I type response. These responses lead to the production of interferon-gamma (IFN-γ) and tumor necrosis factor (TNF), inducing the lysis of infected cells and

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Table 1
Number of cases and viral types, as determined by immunohistochemistry ($n = 68$ cases).

	Total	VSR	AdV	PIV3	PIV2	PIV1	FLU A	FLU B
Number of cases (concomitant viral types)	39	46	26	29	16	11	18	14
Number of cases (pure viral types)	29	11	7	2	4	0	3	2

causing apoptosis [5,7–10]. After natural infection, normal children respond with the proliferation of specific TCD8+ (cytotoxic) lymphocytes, suggesting the stimulation of T cells. Both T lymphocytes, TCD4+ and TCD8+, are involved in RSV replication during infection. In rats, the TCD8+ (cytotoxic) lymphocytes are responsible for the elimination of virus in the lungs. However, paradoxically, they appear to increase symptom severity. The same has been observed with TCD4+ (helper) lymphocytes, which have a protective role and may simultaneously increase disease severity. The predominant stimulation of Th2 lymphocytes may be associated with the occurrence of diseases with increased severity [11–17].

2. Objectives

The aim of this study was to evaluate pneumonic infiltrates by assessing the immunohistochemical expression of cell surface antigens (CD4, CD8, CD25, CD14, CD74).

3. Study design

From cases of pediatric autopsies of patients with severe respiratory infectious diseases, formalin fixed – paraffin embebded lung samples ($n = 193$, 1994–2004) were selected for this study. Stillbirths, neonatal deaths and children with immunodeficiencies were also excluded from the total sample ($n = 794$ — 1960–2004). The ages of the individuals included in this study ranged from one month to 14 years of age, and the cases were divided into two groups: up to 1 year and over 1 year. The dates of the deaths were used to define seasonality, which was divided into warm and cold months. The anatomopathological patterns of the 193 samples of this study were reviewed in sections stained with hematoxylin–eosin and were classified as either bronchopneumonia or interstitial pneumonitis [18,19].

To identify subsets of inflammatory cells by immunohistochemistry, we used the following primary antibodies: anti-CD4 for the identification of T-helper cells (1:20); anti-CD8 for cytotoxic T cells (1:200); anti-CD14 antibody for monocytes, macrophages and histiocytes (1:800); anti-CD25 for the interleukin 2 receptor and the T lymphocyte immune response regulator (1:400); and anti-CD74 for cells expressing MHC class II, including B lymphocytes and macrophages (1:80); anti-human, mouse monoclonal, from Novocastra™ New Castle upon Tyne, United Kingdom.

Tissue microarrays (TMAs) that contained four samples per case, two peribronchial areas and two subpleural peripheral areas, were constructed [18,19]. All TMA slides were stained together with a negative control slide, in which no primary antibody was added, and a positive control slide (human tonsil sample).

For immunohistochemistry, the immunoperoxidase assay, as reported by Chong and colleagues, was used, with modifications. Antigen retrieval was performed using the BioSB®™ imunoretriever (Santa Bárbara, CA, USA). The samples were incubated with the secondary antibody (DAKO ADVANCE™ HRP SYSTEM, DakoCytomation, Inc., CA, USA) for 30 min [18,19].

All cases had already been tested using the same immunohistochemistry protocol described above for respiratory viruses (using the antibodies anti-RSV; anti-AdV; anti-PIV1, PIV2 and PIV3; and anti-FLUA and FLUB) using the LIGHT DIAGNOSTICS™ Respiratory Viral Screen DFA Kit (CHEMICON International, Inc., Temecula, CA, USA) [18,19].

The TMA plates were analyzed with an Olympus BX50 microscope (Olympus Optical Co. LTDA, Japan) at 400× magnification without prior information on the viral positivity of the samples. For each case, 40 high-power fields (HPFs = 400×) were randomly selected and, in each field, the number of positive cells was quantified. The total cells per case (40 HPFs) were used for statistical calculations.

The non-parametric Kruskal–Wallis test was used to compare the two groups with regard to the quantitative variables, and Fisher's exact test or the chi-square test were used to compare the two groups with regard to the categorical variables. P values ≤ 0.05 were considered statistically significant.

4. Results

The most common viral type identified in this study was RSV ($n = 46$). Furthermore, 39 cases had concomitant viral infection, and 16 of them were positive for more than three viruses (Table 1). Regarding the children's sex and age and the seasonality and histopathological patterns, there was no statistical predominance of any group in relation to the presence or absence of virus (see Tables 2 and 3). There was a predominance of cases negative for

Table 2
Distribution of 193 cases according to sex and age range of children, seasonality of infections, pneumonia morphology pattern, presence or absence of virus in the sample by immunohistochemistry and immunophenotyping of pneumonic infiltrates.

Variables	Cases	%
Sex		
Female	86	0.45
Male	107	0.55
Age range		
Up to 1 year of life	119	0.62
>1 year of life	74	0.38
Cause of death		
Bronchopneumonia	54	0.28
Interstitial pneumonitis	10	0.05
Sepsis	70	0.36
Acute respiratory failure	38	0.20
Other	21	0.11
Seasonality		
Cold months	122	0.63
Warm months	71	0.37
Morphology pattern		
Bronchopneumonia	151	0.78
Interstitial pneumonitis	42	0.22
Presence of virus		
No	125	0.65
Yes	68	0.35
Age		
Maximum/minimum value	14 years/1 month	
Median/standard deviation	7 months/2 years and 11 months	
Number of positive cells per high power field		
Median CD4+ cells	8.00	
Median CD8+ cells	12.00	
Median CD14+ cells	36.50	
Median CD25+ cells	1.00	
Median CD74+ cells	79.00	

Table 3
Analysis of positive and negative virus groups with respect to the sex and age of the children, seasonality of infection, pneumonia morphology pattern and immunophenotype of pneumonic infiltrates.

	Total	Positive virus group ($n=68$)		Negative virus group ($n=125$)		p value
		Number	%	Number	%	
Sex						
Female	86	33	48.53	53	42.40	>0.05
Male	107	35	51.47	72	57.60	>0.05
Age group						
Up to 1 year of life	119	44	36.97	75	63.03	>0.05
>1 year of life	74	24	32.43	50	67.57	>0.05
Age						
Median		6 months		7 months		>0.05
Seasonality						
Cold months	122	44	36.07	78	63.93	>0.05
Warm months	71	24	33.80	47	66.20	>0.05
Morphology pattern						
Bronchopneumonia	151	50	33.11	101	66.89	>0.05
Interstitial pneumonitis	42	18	42.86	24	57.14	>0.05
Number of positive cells per high power field						
Median CD4+ cells		10		8		>0.05
Median CD8+ cells		17		9		**0.04**
Median CD14+ cells		21.5		46		0.08
Median CD25+ cells		1		1		>0.05
Median CD74+ cells		76.5		79		>0.05

virus ($n=125$) compared to positive cases ($n=68$), as shown in Table 2.

There were no statistically significant differences between the virus-positive and virus-negative groups in terms of anti-CD4, CD25 and CD74 positivity. However, we found that lymphocytes (TCD8+) were present at significantly higher numbers in the virus-positive group ($p=0.04$). Similarly, results were obtained for CD14+ cells ($p=0.08$), which were present at higher numbers in the virus-negative group (see Table 3).

The results in Table 4 show the comparison between cases of pure RSV ($n=11$) and pure AdV ($n=7$) with the virus-negative group. There were fewer CD14+ cells in AdV-positive cases than in the virus-negative group ($p=0.002$). The counts of positive cells were also analyzed in cases that were positive for pure PIV3 ($n=2$), pure PIV2 ($n=4$), pure FLUA ($n=3$) and pure FLU B ($n=2$). When these cases were compared with the virus-negative group, there were no statistically significant differences between their cell counts.

We also analyzed the counts of positive cells in cases that were positive for combinations of AdV; RSV; PIV1, 2 and 3; and FLU A and B. AdV cases that occurred in association with another virus ($n=26$) had more CD8+ cells ($n=26$) than the virus-negative group ($n=9$; $p=0.0219$) and fewer CD14+ cells ($n=13$) than the virus negative group ($n=46$; $p=0.0269$).

There were no significant correlations between the patients' cell populations and sex, age and seasonality. However, when analyzed in terms of histopathological patterns of lung injury, we observed that patients with interstitial pneumonitis ($n=97$) had greater numbers of CD74+ cells than patients with bronchopneumonia ($n=69$; $p=0.037$).

Table 4
Number of positive cells per high power field in cases of pure VSR and pure AdV, compared with the negative virus group.

	VSR $n=11$	AdV $n=7$	Negative virus group $n=125$	VSR p value	AdV p value
CD4+	21	13	8	0.123	0.981
CD8+	8	27	9	0.880	0.211
CD14+	42	5	46	0.735	**0.002**
CD25+	0	1	1	0.566	0.675
CD74+	91.5	34	79	0.452	0.108

Furthermore, patients with more than 3 viruses ($n=38$) had greater numbers of TCD8+ lymphocytes than patients with up to 3 viruses ($n=10$; $p=0.016$).

5. Discussion

The sex distribution of the population studied showed a slight predominance of males over females. In studies in developed and developing countries, more males than females are affected by respiratory infections [4,7,18–21]. In our study sample, 62% ($n=119$) were children up to 1 year of age as has been previously reported in the literature [18,19,22]. There was a predominance of deaths in the colder months (63%) compared to the warmer months (37%). Of the respiratory viruses, RSV showed the most characteristic seasonality, which has already been well documented in studies in developed and developing countries [18,19,23]. Bronchopneumonia was the most common histopathologic pattern, representing 78% of the total sample. Classically, bronchopneumonia and lobar pneumonia are histopathological patterns described in lung infections caused by bacteria. However, numerous studies have shown that respiratory viruses alone or concomitant viral-bacterial infections can also be etiologic agents of these findings. Thus, when a patient's morphologic pattern is characteristic of the bacterial etiology of lung infections, the existence of viral concomitance is also likely in these cases, as it is believed that respiratory viruses can favor the entry and spread of bacteria, both locally and systemically [18,19,24].

In this study, 68 cases were positive for viral antigens, resulting in a 35% prevalence of cases positive for virus by immunohistochemistry. Studies with other methods, such as indirect immunofluorescence, corroborate this result and have reported viral positivity in acute respiratory childhood infections ranging from 31.9 to 56.4%. In the analysis of viral types, RSV was the most commonly identified virus. RSV was also the most common agent identified in other studies and has been reported to cause severe pneumonia in the United States, Italy, South Korea, Uruguay and Brazil [6,11,18,19,25,26].

In situ hybridization or immunohistochemistry analysis of respiratory infections has demonstrated the presence of virus in bronchial epithelial cells and single alveolar epithelial cells, especially in recent infections (3 days of hospitalization). The lack

antigen detection of viruses in alveolar epithelial cells may be related to the long time course of these infections. In this study, we observed bronchial, bronchiole and alveolar epithelial cells, positive for viral antigens by immunohistochemistry in samples from patients who died an average of 13 days post hospitalization [17,18].

Besides the limited amount of tissue that is analyzed by TMA, this technique offers many benefits, such as the uniformity of staining reactions, and also helps to prevent necrotic and hemorrhagic areas, which may complicate immunohistochemistry assessment [17,18].

It is important to mention that we only looked at these seven viruses in this study, and important pediatric respiratory viruses (rhinovirus, metapneumovirus, coronaviruses), were not analyzed. Clearly, inclusion of the latter viruses in our study may have increased the number of positive samples [17,18].

The immunophenotyping of 193 samples revealed a predominance of CD74+ cells (median of 79), with no significant difference between virus-positive and virus-negative cases. Moreover, the CD14+ cell counts were also high (median of 36.5) in all samples, in contrast to CD25+ cell counts, which were few in number in all of the study samples (median of 1). Additionally, the number of CD25+ cells was not significantly different between the virus-positive and virus-negative groups. However, CD4+ cells (lymphocytes and some macrophages) were detected in all cases (median of 8), and no statistically significant differences were observed when comparing the virus-positive and virus-negative groups. These differences between the numbers of cell types are due to the different presentations of pneumonia and pathological reactions of the host, along with the differences in etiologic agents, length of hospitalization and duration of illness [18,19].

One of the reasons for the observations of high numbers of cells expressing CD74 and CD14 is the fact that most cases in this study involved patients with bronchopneumonia, which was most likely purely bacterial or concomitantly viral. In our samples, the cells that expressed CD74, an MHC class II marker, were mainly B lymphocytes, histiocytes and macrophages, while those cells that expressed CD14, a general monocyte marker, were mainly histiocytes and macrophages. Bronchopneumonia, which is usually bacterial, has a lesion pattern that is mainly characterized by neutrophilic exudation. Neutrophils are attracted to the focus of infection through chemotactic cytokines released by immune cells, mainly macrophages and B lymphocytes, which generally differentiate subsequent to MHC class II bacterial antigen presentation. Moreover, by immunohistochemistry, most cases did not show viral markers, and the bronchopneumonia pattern was predominant. This observation leads us to believe that these cases had been treated for pure bacterial pneumonia. This is characterized by high concentrations of neutrophils in the acute phase, an inflammatory response mainly mediated by MHC class II, and a resolution phase mediated by CD14+ and CD74+ histiocytes. Another interesting point is that as these were cases of patients who had already died, the hospitalization time was relatively high (average of 13 days), regardless of the histopathological pattern found. Accordingly, in these patients, standard pulmonary pathology that was already in the resolution phase, with large amounts of CD14+ and CD74+ histiocytes, was common. This observation would explain the overall prevalence of these markers in this study, even though the differences between the groups with or without virus were not statistically significant [6,11–13,26–28].

CD25+ cells are generally rare in inflammatory processes because they function as regulators of lymphocytes in the immune response, which would explain their low numbers observed in our samples. Additionally, there were no significant differences between the virus-positive and virus-negative groups because they modulate both Th1 and Th2 responses [13].

The median number of CD4+ cells was low in all cases (8 CD4+ cells by HPF) and did not present statistically significant differences. These cells are usually T-helper lymphocytes of both the Th1 and the Th2 immune responses; therefore, they were present both in cases of pneumonitis, which skews toward the Th1-type lymphocyte response, and in cases of bronchopneumonia, which skews toward the more neutrophilic Th2 response [13].

As observed by HPF, the mean number of TCD8+ lymphocytes in the total sample was 12, and there were statistically significant differences in counts when comparing virus-positive cases (17 TCD8+ lymphocytes by HPF) with virus-negative cases (9 TCD8+ lymphocytes by HPF); virus-positive cases presented higher numbers of TCD8+ lymphocytes, as did those cases with more than 3 positive viruses ($p = 0.04$ and $p = 0.016$, respectively). Respiratory viruses are capable of inducing hyper-responsiveness in the airways by activating both Th1 and Th2 responses, with the Th1 response usually being predominant. Accordingly, staining with anti-CD8 antibody revealed a greater number of TCD8+ lymphocytes in virus-positive cases, indicating the prevalence of the activation and proliferation of cytotoxic T lymphocytes, suggesting a Th1-type immune response. There is controversy in the literature regarding the immune response mediated by TCD8+ lymphocytes. Other studies have shown that the Th1 response is of fundamental importance in RSV pathogenesis. Subsequently, Thorburn [29] showed that RSV inhibits the expression of TCD8+ lymphocytes in the pulmonary parenchyma and the development of TCD8+ memory cells, as RSV seems to interfere with receptor signaling in these cells. Corroborating these data, in this study, we also found that there were large numbers of TCD4+ lymphocytes and CD4+ macrophages in lung samples, indicating the involvement of the Th2 response. Such an immune response mechanism may be due to the predominance of positive RSV cases in this study, which could have interfered with the final counts of these inflammatory cells for the virus-positive group.

Thus, these differences were further assessed by analyzing the cases of pure RSV [13,29,30], as shown in Table 4. Specifically, compared to pure RSV cases, negative cases had fewer TCD4+ and CD74+ cells and mild increased numbers of CD14+ and CD8+cells. In the cases of pure AdV compared to negative cases, there was a reduction in TCD8+ and CD4+ lymphocyte counts and an increase in CD14+ ($p = 0.002$) and CD74+ cells in negative group.

These data suggest that cases of AdV may have contributed to the increased numbers of TCD8+ lymphocytes in cases with viral positivity. These facts could also suggest that the pulmonary inflammatory processes caused by AdV involve the activation of the Th1 response and the inhibition of the Th2 response, as previously suggested by other authors [6,29–31].

The results of this study demonstrate that in cases of viral pneumonia, there are a greater number of TCD8+ lymphocytes present in the alveolar septa. Additionally, in cases of AdV, there are fewer numbers of CD14+ cells.

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Competing interest

None declared.

Ethical approval

The ethics review board of the HC-UFPR reviewed and approved the study. Register number 1099.138/2005; August 30, 2005.

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